# MOLECULAR ISOLATION AND CHARACTERIZATION OF TUMOR NECROSIS FACTOR (TNF) GENE OF *Macrobrachium rosenbergii*

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# การแยกและการศึกษาคุณสมบัติของยีน tumor necrosis factor จากกุ้งก้ามกราม (*Macrobrachium rosenbergii*)

พรฉัตร สุขเสงี่ยมกุล

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# MOLECULAR ISOLATION AND CHARACTERIZATION OF TUMOR NECROSIS FACTOR (TNF) GENE OF *Macrobrachium rosenbergii*

PHORNCHATRA SUKSANGIAMKUL

A Thesis Submitted in Partial Fulfillment of the Requirements

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MOLECULAR ISOLATION AND CHARACTERIZATION
OF TUMOR NECROSIS FACTOR (TNF) GENE
OF *MACROBRACHIUM ROSENBERGII*

BY

PHORNCHATRA SUKSANGIAMKUL

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IN BIOTECHNOLOGY AT SRINAKHARINWIROT UNIVERSITY

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| Title | MOLECULAR ISOLATION AND CHARACTERIZATION OF TUMOR NECROSIS FACTOR (TNF) GENE OF *Macrobrachium rosenbergii* |
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The tumor necrosis factor (TNF) is a cytokine that plays important roles in various physiological pathways, including inflammation and immune responses to microbial infections in vertebrates. TNF functions by binding to tumor necrosis factor receptor (TNFR) and initiating the signaling cascade to activate the NF-**K**B pathway and activator protein 1 (AP-1) pathway, which are required for the expression of pro-inflammatory cytokines. However, the role of TNF in immune responses in invertebrates remains largely unknown. Therefore, in this study, the full-length TNF gene of *Macrobrachium rosenbergii* (*MrTNF*) was isolated and characterized, then the expression of *MrTNF* in response to *Aeromonas hydrophila* and *Mr*NV infections was investigated. The full-length cDNA of *MrTNF* was 1830 base pairs (bp), consisting of 5' untranslated region (5'-UTR) of 396 bp and 3'-UTR of 54 bp. *MrTNF* contained an open reading frame (ORF) of 1380 bp encoding 459 amino acid residues. The *Mr*TNF structure revealed the presence of a transmembrane domain at positions 21-43 and a conserved TNF domain at positions 324-446. The *Mr*TNF protein revealed 91.88% identity with *Mn*TNF from *Macrobrachium nipponense*. The expression level of *MrTNF* mRNA in healthy prawns exhibited high expression in the intestine, muscle, and stomach. *MrTNF* was significantly up-regulated in hemocyte, muscle, intestine, and stomach upon *A. hydrophila* infection. Furthermore, the expression level of *MrTNF* in muscle, gills, and hepatopancreas was significantly up-regulated upon *Mr*NV challenge. These results indicated that *Mr*TNF may play a crucial role in the innate immune system of freshwater crustaceans in response to bacterial and viral infections.

Keyword : Macrobrachium rosenbergii, Giant freshwater prawn, Innate immunity, Tumor necrosis factor, TNF

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PHORNCHATRA SUKSANGIAMKUL

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# CHAPTER 1
# INTRODUCTION

## Background

*Macrobrachium rosenbergii* is a species of freshwater prawn which is also commonly known as giant freshwater prawn or giant river prawn. The natural range of this species is found in Indo-West Pacific from Northwest India to Australia, Bangladesh, Brunei Darussalam, Cambodia, China, Indonesia, Malaysia, Myanmar, New Guinea, Pakistan, Philippines, Singapore, Sri Lanka, Thailand, Vietnam, and also introduce to Brazil and Martinique island (De Grave, Shy, Wowor,& Page, 2013; Palomares, 2020). Giant freshwater prawn is widely cultured in the natural range and also distributed to Africa, Europe, North America, and South America. Food and Agriculture Organization (FAO) has recorded the status of global aquacultural production, giant freshwater prawn being major species product in aquaculture that has been exceeded to 313.7 thousand tonnes which accounted the global value of over USD 2.45 billion in 2021 (FAO, 2023). In Thailand, the giant freshwater prawn aquaculture was established by the co-project of FAO with the United Nations Development Programme (UNDP), called "Expansion of Freshwater Prawn Farming in Thailand". This project was proceeded together by the Department of Fisheries (DOF) Thailand in 1977, which led to the giant freshwater prawn aquaculture become widely well-known for over decades (New, 2000). The success of giant freshwater prawn production has been grown from 5 tonnes in 1976 to 400 tonnes in 1981. Currently, Thailand's DOF has released the statement of freshwater prawn production about 43.4 thousand tonnes with an economic value of USD 239.4 million in 2021 (Fisheries Development Policy and Planning Division, 2020; 2022). However, giant freshwater prawn production in Thailand has tended downward during 2009 to 2021. Farmers have been suffered from many problems including overproduction, low-profit margins, high costs, and especially, infectious diseases caused by bacteria such as *Aeromonas hydrophila*, *A. veronii*, *A. caviae*, *Vibrio harveyi* and *V. parahaemolyticus* (Rojtinnakon, Promya, & Klairuang, 2009), viruses including *Macrobrachium* Hepatopancreatic Parvo-Like Virus (MHPV), *Macrobrachium rosenbergii* nodavirus

(*Mr*NV), Extra Small Virus (XSV), and White Spot Syndrome Virus (WSSV), fungi *Candida mogi*, *Fusarium solani*, protozoan including *Corthunia* sp., *Epistylis* sp., and *Vorticella* sp., metazoan *Digenean Metacercariae*, and parasitic pathogens, microsporidia (Fisheries Development Policy and Planning Division, 2020; Farook, Mohamed, Tariq, Shariq, & Ahmed, 2019; Hooper et al., 2023; Sudthongkong, n.d).

The infectious disease outbreak leads to a high mortality rate and economic loss in giant freshwater prawn aquaculture. The knowledge of fundamental research in giant freshwater prawn biology especially, the immune system may be able to develop farming to be sustainable with less of environmental impacts. Therefore, understanding of the functions of genes and proteins that involved in shrimp immune system is essential to control the infectious diseases.

According to the lack of an adaptive immune system in shrimp, the innate immune system plays a crucial role in defending against pathogen infections. The innate immune cells rapidly respond to invading microbes by pattern recognition receptors (PRRs) which can recognize the pathogen-associated molecular patterns (PAMPs) on the surface of pathogen. The innate immune system consists of both humoral and cellular immune responses, with the humoral immune response acting as the primary defense mechanism against pathogen infections. Upon detecting extracellular signaling molecules from the pathogen such as PAMPs or viral protein, PRRs will be initiated signaling transduction, which triggers the activation of the nuclear factor kappa B (NF-$\kappa$B) signaling pathways for synthesis and secretion of various immune proteins such as antimicrobial peptides (AMPs), cytokine-like factors, melanization, phenoloxidase, and proteinase inhibitors (Tassanakajon et al., 2018), whereas the cellular immune response involves in apoptosis, phagocytosis, nodulation, and encapsulation of invading pathogens. The major mechanism occurs in hemocyte which involves in synthesis of humoral effectors. (Tassanakajon, Somboonwiwat, Supungul, & Tang, 2013). Nowadays, there are several studies focus on genes and proteins that play an essential role in shrimp immune system such as Toll signaling pathway, Immune deficiency (IMD) pathway, Janus kinase/signaling transducers and activators of transcription (JAK/STAT)

pathway, to prevent the invading pathogens (Li & Xiang, 2013b). Nevertheless, understanding of the mechanisms involves in the immune response remains incomplete.

Tumor necrosis factor (TNF) is a cytokine that belongs to the tumor necrosis factor superfamily (TNFSF). TNF plays a crucial role in immune responses by binding to the tumor necrosis receptor (TNFR) and initiating the signaling cascade to activate the NF-$\kappa$B pathway and activator protein 1 (AP-1) pathway, both of which are essential for the production of pro-inflammatory cytokines to induce cell inflammatory, cell proliferation, cell survival, and activate caspase enzyme that leads to cell apoptosis (Chu, 2013; Wajant, Pfizenmaier, & Scheurich, 2003). In invertebrates, TNF and TNFSF have been identified and characterized from various species. In fruit fly, *Drosophila melanogaster*, there are two proteins named Eiger and Wengen that belong to the TNFSF and TNFR superfamily, respectively. Eiger can bind to Wengen and lead to activation of signaling transduction to the c-Jun N-terminal kinase (JNK) pathway for inducing cell apoptosis (Igaki et al., 2002; Kauppila et al., 2003; Moreno, Yan, & Basler, 2002). TNFSF members have been discovered in four shrimp species, which include *Marsupenaeus japonicas* (*Mj*TNF), *Litopenaeus vannamei* (*Lv*TNFS, *Lv*TNFRSF, and *Lv*LITAF), *Procambarus clarkii* (*Pc*TNF), and *Macrobrachium nipponense* (*Mn*TNF). The *Mj*TNF shared 30.7% identity with *Drosophila* Eiger. The *Mj*TNF expression was upregulated after stimulation with *V. penaeicidae*, and the result suggested that *Mj*TNF may response to Gram-negative bacteria (Mekata et al., 2010). In *L. vannamei*, *Lv*TNFSF showed 89.8% identity with the *Mj*TNF and *Lv*TNFRSF showed identity of 14.6% with *Drosophila Wengen*. *Lv*TNFSF expression was increase in response to *Staphylococcus aureus*, WSSV, and *C. albicans* infections, this indicated that *Lv*TNFSF may be involved in the immune response against various pathogens, especially Gram-positive bacteria, virus, and fungi (Wang et al., 2012). In *M. nipponense*, *Mn*TNF showed 76% and 38% identity with *Mj*TNF and *Drosophila* Eiger, respectively. The *Mn*TNF expression was observed by RNA interference (RNAi) mediated *Mn*TNF silencing after challenged with *A. veronii*. The result suggested that *Mn*TNF could enhance the expression of AMPs gene, crustin. In addition, the expression of NF-$\kappa$B transcription factor gene, relish, was

considerably up-regulated. Therefore, *Mn*TNF may involve in immune response by regulating some of AMPs genes (Qin, Tang, Liu, Xie, & Liu, 2019).

In the past, there were several studies tried to investigate the host-pathogen interaction through molecules responsible for innate immune system of giant freshwater prawn. For example, Toll receptor (*Mr*Toll) has been reported to be up-regulated *after A. caviae* challenge (Srisuk, Longyant, Senapin, Sithigorngul, & Chaivisuthangkura, 2014). The ligand for the Toll receptor in the Toll signaling pathway, known as Spätzle (*Mr*Spz) was found to be activated in response to *A. caviae* infection. (Vaniksampanna, Longyant, Charoensapsri, Sithigorngul, & Chaivisuthangkura, 2019). The NF kappa B inhibitor alpha (*Mr*NF-$\kappa$BI-$\alpha$) exhibited up-regulation in hemocytes after *A. hydrophila*, *Enterococcus faecium*, infectious hypodermal and haematopoietic necrosis virus (IHHNV) and polyinosinic:polycytidylic acid (poly I:C) infections (Arockiaraj et al., 2012). The PRRs, C-type lectin (*Mr*CTL) with a single carbohydrate recognition domain (*Mr*CRD) was exhibited the agglutinating and binding activities against several bacterial infection and also in the recombinant *Mr*CTL that was found to enhance the clearance of *V. parahaemolyticus* in prawns (Huang, Feng, Jin, Ren, & Wang, 2016).

Currently, there is no report about the tumor necrosis factor (TNF) in giant freshwater prawn. Therefore, this research is the first study aimed to isolate and characterize the full-length *TNF* gene of *M. rosenbergii* (*MrTNF*). The molecular characterization of *Mr*TNF and its phylogenetic relationship with other TNF homologs were investigated. Additionally, the distribution of *MrTNF* was examined among various tissues of *healthy M. rosenbergii* and investigated the expression of *MrTNF* against *A. hydrophila* and *Mr*NV infections. The characterization of *Mr*TNF may lead to further understanding of the role in innate immune response in *M. rosenbergii* against bacterial and viral infections.

## Objectives

1. To clone and characterize the full-length of *TNF* gene from *M. rosenbergii* (*MrTNF*)
2. To examine the gene expression patterns of *MrTNF* in various tissues of healthy prawns.
3. To investigate the expression of *MrTNF* against bacterial and viral infection.

## Hypotheses

1. *MrTNF* is hypothesized to be considerably conserved among various TNFs proteins.
2. The expression of *MrTNF* is expected to be highly expressed in certain tissue organs.
3. The expression of *MrTNF* is anticipated to be triggered by *A. hydrophila* and *Mr*NV infection.

## Scopes

1. Cloning of *MrTNF* using rapid amplification of cDNA ends (RACE).
2. Characterization of *Mr*TNF using bioinformatics tools.
3. Construct the phylogenetic tree of *Mr*TNF among TNFs from various species.
4. Analysis of the gene expression patterns of *MrTNF* in several tissues of healthy prawns and *A. hydrophila* and *Mr*NV infected prawns using quantitative real-time RT-PCR (qRT-PCR).

# CHAPTER 2
# LITERATURE REVIEW

## 1. Giant freshwater prawn (*Macrobrachium rosegenberii*)

*M. rosenbergii* has a common name as giant freshwater prawn or giant river prawn and other local names may be used in different countries. For instance, in Thailand, it is called Koong Ghram Gram (Brown, 2019; Holthuis, 1980; Repotente, 2008). *M. rosenbergii* is an important commercial aquatic species worldwide, mostly in Asia and South America. The global production of *M. rosenbergii* has exceeded from 217 thousand tonnes in 2010 to 238 tonnes in 2016 and has continuously achieved high production for over a decade (FAO, 2020). In Thailand, farming of *M. rosenbergii* is widely distributed to several freshwater inland such as Nakhon Prathom, Ratchaburi, Chachoengsao, Kalasin, and Suphan Buri. The production over 9,000 tonnes of *M. rosenbergii* in 2021-2022 with the market value of USD 3.6 million were exported to the USA, Myanmar, and Malaysia. For this reason, Thailand has become the major producer of *M. rosenbergii* in the world (Fisheries Development Policy and Planning Division, 2023).

**Taxonomic classification of *M. rosenbergii***

Domain: Eukaryota

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Subclass: Eumalacostraca

Order: Decapoda

Suborder: Natantia

Family: Palaemonidae

Genus: *Macrobrachium*

Species: *Macrobrachium rosenbergii*

**Biological characteristics and morphology**

*M. rosenbergii* is the largest species among the *Macrobrachium* genus, with a body length reaching up to 320 mm in adult male and up to 250 mm in adult female. In general, typical of a decapod crustacean, the body of post larvae (PL) and adult prawns comprises the cephalothorax and the abdomen. The cephalon and thorax is merged into the cephalothorax. The cephalon or head are covered by the carapace, which contains of an eye, rostrum, five appendages; antennules or the first antenna, antennae or the second antennae, mandibles, maxillules or the first maxillae, and maxillae as the second maxillae. The thorax of prawn comprises eight pairs of appendages which contains three pairs of mouthparts called maxillipeds I, II, and III, and five pairs of true legs known as pereopods. The abdomen has six somites, which contains of five pairs of pleopods (swimmerets), telson (tail fan), and pairs of uropod (New, Valenti, Tidwell, D'Abramo, & Kutty, 2010). *M. rosenbergii* has been distinguished from other species by the special characteristics such as the long rostrum located at the front of the cephalon, which has 11 to 14 teeth on the dorsal side and 8 to 10 teeth on the ventral side. Additionally, the first pair of pereopods are tightly folded up under the cephalon and function as feeding appendages with fine forcep-like chelae at their tips. In adult males, the second pereopods (chelipeds) are very long, and the mobile finger of chelipeds is covered with velvet-like fur while the fixed finger is missing (Brown, 2019; New, 2002; New et al., 2010). *M. rosenbergii* usually has a body color vary from greenish to brownish grey that can be more bluish sometimes. The lateral ridge of rostrum may appear in red and orange while the vertical ridge is blue. The antennae and the chelipeds are often bluish as presented in Figure 1 (New, 2002; New et al., 2010). The morphology of *M. rosenbergii* is presented in Figure 2.

Figure 1 Giant freshwater prawn *(M. rosenbergii)*

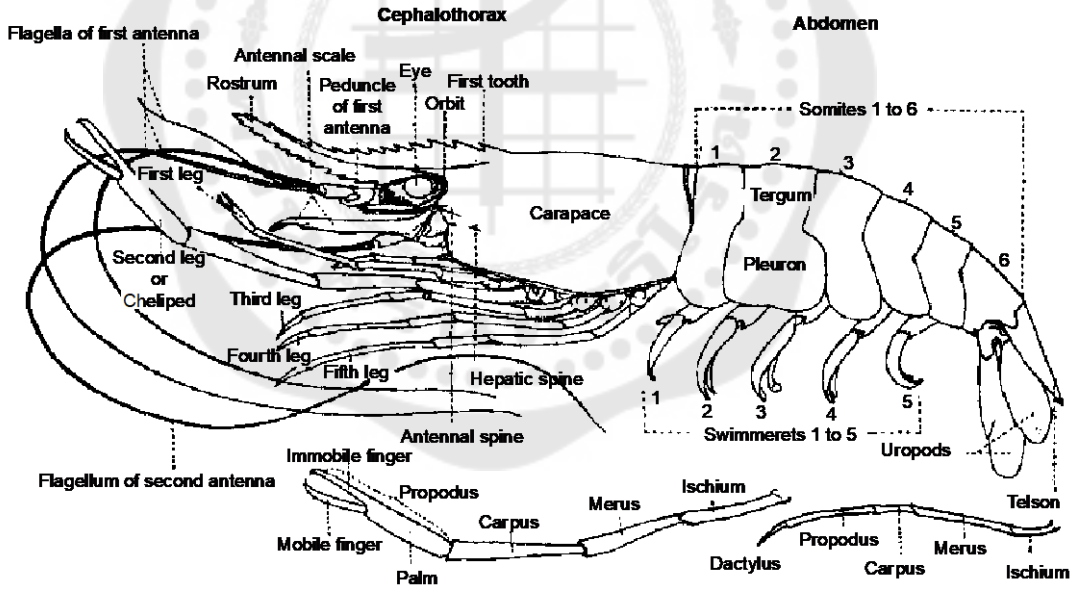



Figure 2 Morphology of *M. rosenbergii*

Source: New, M. B., Valenti, W., C. Tidwell, J. H., D'Abramo, L. R., & Kutty, M. N. (2010). *Freshwater prawns : biology and farming*: Blackwell Publishing Ltd.

**Habitat and life cycle**

*M. rosenbergii* is distributed in the tropical and subtropical regions across the world, mostly in freshwater inland environments such as lakes, rivers, swamps, irrigation ditches, canals, and also in estuaries (New, 2002). The ecological niche of the water temperature is about 25-34°C and wide range of salinity is about 0-20 ppt. *M. rosenbergii* is active in nocturnal although, prawns is shaded and shelters during the day (Kadam, 2021). In breeding season, the matured female prawns will be molting while the adult male prawns have hard shell. The adult male prawns deposit a spermatophore into the underside of the female's thorax. The matured male prawns are usually larger than the matured female prawns (Chowdhury, Bhattacharjee, & Angell, 1993; Ling, 1967). The gravid females have migrated downstream into estuaries water for hatching as free-swimming larvae. Before undergoing metamorphosis into PL, *M. rosenbergii* planktonic larvae progress through several zoea stages, ranging from zoea I to zoea XI. After metamorphosis, PL begin migrating upstream into freshwater (New et al., 2010). The life cycle of *M. rosenbergii* is presented in Figure 3.

The larvae mostly consume small worms, zooplankton, and larvae of other crustaceans. The post larvae and mature prawns are omnivorous, which feed on an algae, aquatic plants, aquatic insects, worms, and other crustaceans (New et al., 2010).

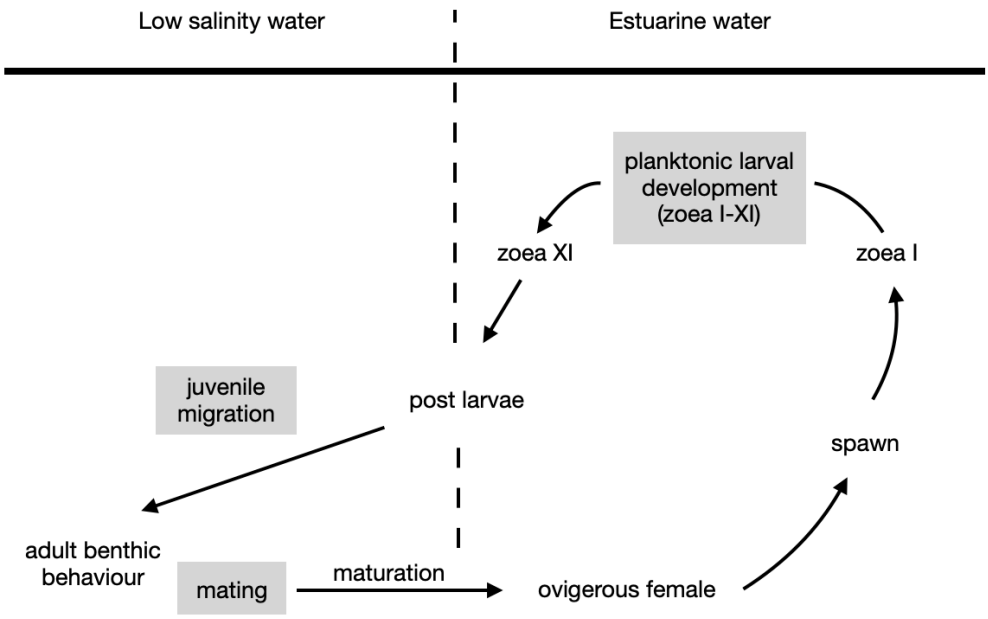



Figure 3 Life cycle of *M. rosenbergii*

Source: Modified from New, M. B., Valenti, W., Tidwell, J. H., D'Abramo, L. R., & Kutty, M. N. (2010). *Freshwater prawns: biology and farming*: Blackwell Publishing Ltd.

**Diseases**

The problem of infection and disease in *M. rosenbergii* farming is a major concern in the aquaculture industry, in terms of prawn production and causing economic losses. *M. rosenbergii* can be affected by various pathogenic microorganisms such as bacterium, fungi, and viruses. The pathogen can cause disease outbreaks that lead to high mortality rates among infected individuals, which impact on the population size and the genetic diversity of the prawn species. Furthermore, the pathogenic diseases can also affect the quality and safety for human consumption. Therefore, it is important to take a preventive measures and implement effective disease management strategies to minimize the risks of pathogenic disease outbreaks in *M. rosenbergii*.

*Aeromonas hydrophila* is a Gram-negative bacteria, with rod-shaped typically 1-4 μm in size, non-spore-forming, motile with a flagellum, and lacking pigment and capsule. *A. hydrophila* is a facultative anaerobe commonly found in freshwater environments (Rojtinnakon et al., 2009). The virulence factors present in *A. hydrophila* have been identified as haemolysin, aerolysin, cytosine, and enterotoxin (Zeng, 2020). The infection of *A. hydrophila* is emerging as a critical risk factor in commercial aquaculture due to the wide range of fish and shellfish species that have been reported to be sensitive to this infection. In *M. rosenbergii*, the Infection with *A. hydrophila* causes a disease known as “Shell disease or Black spot disease”. The clinical signs of infection include black spots, dark brown marks, or black streaks on the shell surface around the head, body, tail, and appendages of the prawn as presented in Figure 4 (Thancharoen, 2019). Additionally, the histopathological signs include tissue melanization, focal necrosis, haemocyte infiltration, and hyperplasia in the gills, hepatopancreas, and heart as presented in Figure 5 (Abdolnabi, Ina-Salwany, Daud, Mariana, & Abdelhadi, 2015). Another common symptom is that the prawns become more aggressive and tend to fight smaller or weaker prawns, leading to increased high mortality rate and loss of reproduction (Rojtinnakon et al., 2009). In recent years, outbreaks of *A. hydrophila* infection in *M. rosenbergii* have been reported in several countries such as Brazil, India,

Malaysia, Taiwan, and Thailand (Abdolnabi et al., 2015; Petjul, Kulvitit, Tankrathok, & Suebchompoo, 2018; Sahoo et al., 2007).

*Macrobrachium rosenbergii* nodavirus (*Mr*NV) is a virus that belongs to the family Nodaviridae, a small icosahedral virus with a diameter of approximately 26-27 nm (Yoganandhan, Leartvibhas, Sriwongpuk, & Limsuwan, 2006). The virus contains two positive-sense RNA, single strand RNA (ssRNA) which consist of RNA1 encodes for the RNA-dependent RNA polymerase (RdRp) and B2-like protein, while RNA2 encodes for the capsid protein (Ganesan et al., 2022). *Mr*NV is known to exist in both fresh and brackish water. *Mr*NV primarily infects in the freshwater prawn *M. rosenbergii*, along with co-infection virus, extra small virus (XSV), causing a disease known as "White Tail Disease (WTD)" (Chen et al., 2021). The disease is characterized by clinical sign such as whitish muscle of the abdominal, anorexia, lethargy, and high mortality of infected prawns (Murwantoko, Bimantara, Roosmanto, & Kawaichi, 2016). *Mr*NV replicates in the cytoplasm of cells throughout almost all parts of the tissue organ, leading to muscle necrosis and cell death in the abdominal segment, head, thorax, tail, appendages, and the connective tissue in hepatopancreas (Chen et al., 2021; Wannapat, 2019). The histological signs of *M. rosenbergii* infection with *Mr*NV are reported to be severe muscular necrosis and myolysis as presented in Figure 6 (Jang et al., 2022). In *M. rosenbergii*, WTD typically exhibits symptoms of muscle atrophy that appear approximately 10 days after the post-larval stage. The mortality rate is high, ranging from 50-90%, and usually occurs on 4-5 days after infection. In severe cases, mortality rates can reach as high as 90% within 2-3 days (Wannapat, 2019). However, in their adult stages, they can develop resistance to the disease (Jang et al., 2022). Currently, WTD in *M. rosenbergii* has been reported in many countries, including Australia, China, India, Indonesia, Malaysia, Taiwan, Thailand, and South Korea (Ganesan et al., 2022; Jang et al., 2022; Yoganandhan et al., 2006).

Figure 4 Clinical sign of *M. rosenbergii* infected by *A. hydrophila*.
Red arrow is shown the symptom of back spot.

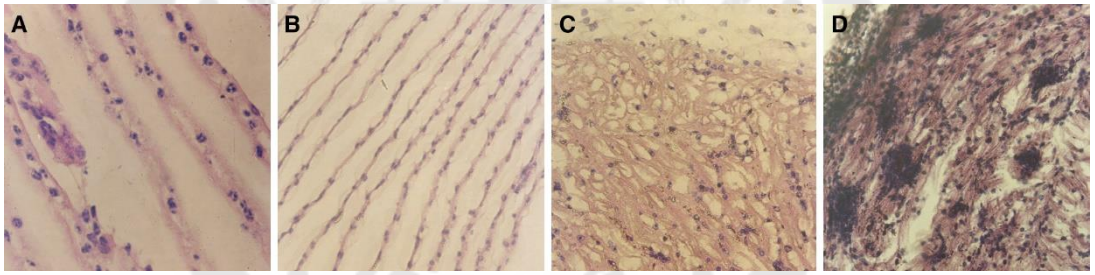



Figure 5 Histological changes of the *M. rosenbergii* infected by *A. hydrophila*.
Gill lamella of the healthy prawn (A). Gill lamella of *A. hydrophila* infected prawn showed a massive infiltration of haemocytes and hyperplasia of epithelial cells (B). Heart musculature of the healthy prawn (C). Melanization in the heart of *A. hydrophila* infected prawn (D). H & E staining and 200x magnification

Source: Modified from Sahoo, P. K., Pillai, B. R., Mohanty, J., Kumari, J., Mohanty, S., & Mishra, B. K. (2007). In vivo humoral and cellular reactions, and fate of injected bacteria *Aeromonas hydrophila* in freshwater prawn *Macrobrachium rosenbergii*. *Fish & Shellfish Immunology, 23*(2), 327-340.

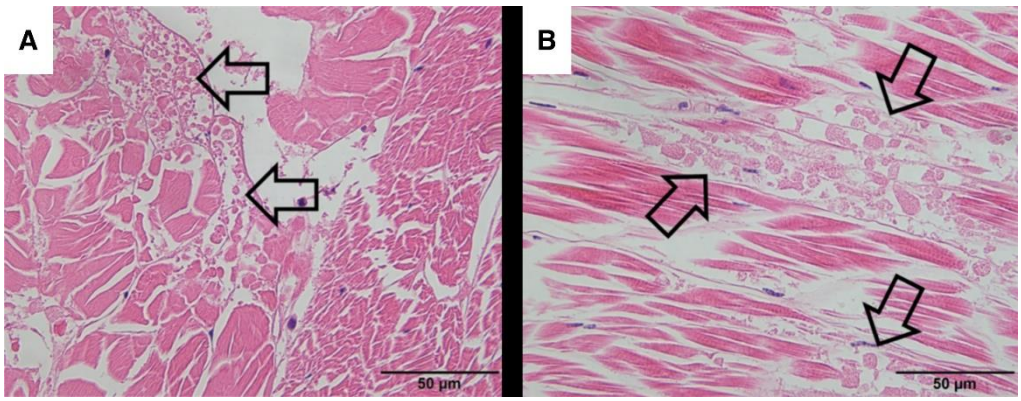



Figure 6 Histological sign of the abdominal muscle of *M. rosenbergii* infected by *Mr*NV. The local myolysis indicated by transparent arrow (A and B) with H&E staining.

Source: Modified from Jang, G. I., Kim, B. S., Kim, S. M., Oh, Y. K., Kim, J. O., Hwang, J. Y., . . . Kwon, M. G. (2022). Detection of *Macrobrachium rosenbergii* Nodavirus (*Mr*NV) of White Tail Disease (WTD) in Apparently Healthy Giant Freshwater Prawn, *Macrobrachium rosenbergii* in Korea. *Fishes, 7*(5), 294.

## 2. Shrimp immune system

Crustaceans and other invertebrates lack an adaptive immune system. The immune system of shrimp primarily relies on the innate immune system as the defense mechanism against invading pathogen upon non-specific immune response but rapidly response to pathogen through activation of pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) or viral components. The innate immune system consists of the cellular immune response and the humoral immune response which triggers responsive via signal transduction pathways such as Toll pathways, IMD pathways, and JAK/STAT pathways (De Gregorio, Spellman, Tzou, Rubin, & Lemaitre, 2002; Li & Xiang, 2013b).

The cellular immune response can be triggered immediately by phagocytosis, apoptosis, nodule formation, encapsulation, and RNAi. The major reply of cellular response has been in the hemocytes, which consist of three different types, named as hyaline, granular and semigranular hemocytes. Each cell type has specific or related functions. For example, hyaline cell or agranulocyte is mainly responsible for phagocytosis, while the granular cell involves in prophenoloxidase (proPO) system and storage of the cytotoxicity. The semigranular cells is participates mainly in encapsulation in addition to phagocytosis, proPO system, which consists of several proteins that involve in melanization and cytotoxicity response. Moreover, the hemocytes also involve in the major synthesis of the humoral effector molecules (Jiravanichpaisal, Lee, & Söderhäll, 2006; Johansson, Keyser, Sritunyalucksana, & Söderhäll, 2000).

The humoral immune response is mediated by the molecules in hemolymph which are activated and secreted from hemocytes. The hemolymph contains several biochemical molecules, such as antimicrobial peptides (AMPs), agglutinin, cytokine-like, lectin, and lysozyme. The highlight of humoral immune response is the melanization by phenoloxidase enzyme (PO) released from proPO system, blood clotting system, and triggers the signaling pathways to secrete the activation of humoral molecules to get rid of the pathogen infection (Tassanakajon et al., 2018). Several PRRs have been identified in shrimp, which play a crucial role in initiating the proPO system. For example, β-1,3-

glucanase-related protein (BGRP), lipopolysaccharide and β-1,3-glucan binding protein (LGBP), C-type lectins, immunoglobulin-related proteins, toll, and tetraspanin (Li & Xiang, 2013a; Wang & Wang, 2013)

**The immune signaling pathway**

The signaling transductions are triggered by recognition of pathogens by the PRRs. These mechanisms are activated when the pattern recognition proteins (PRPs) recognize the pathogen-associated molecular patterns (PAMPs) on microbial surface, for example, β-1,3-glucan, lipopolysaccharides (LPS), and peptidoglycan (PGN) (Vazquez et al., 2009). The downstream signaling leads to synthesize and release of several AMPs or other immune proteins that directly response to invading pathogen. The main signaling pathways in the innate immunity of shrimp include Toll pathway and IMD pathway (Li & Xiang, 2013b) whereas other pathways can be indirectly responded such as JAK/STAT pathway and JNK pathway (Tassanakajon et al., 2018).

The Toll pathway is significant in crustaceans as it is involved in response to Gram-positive bacteria, Gram-negative bacteria, and viruses (Li, Wang, & He, 2019). The signaling cascade of Toll pathway is initiated by the extracellular ligand, Spätzle that is transmitted to cytosol molecules via their adaptor protein, myeloid differentiation factor 88 (MyD88). This leads to the activation of the NF-$\kappa$B transcription factor, Dorsal, which migrates into the nucleus to regulate and synthesize the cognate AMPs, such as crustin and ALFs (Feng et al., 2016; Vaniksampanna et al., 2019). The IMD pathway can directly recognize Gram-negative bacteria and viral infection by their transmembrane receptors. The signaling cascade relies on 2 routes for activation of the NF-$\kappa$B transcription factor, Relish. The first cascade, phosphorylation of Relish, occurs through the IKK complex (Wang et al., 2013) whereas the second cascade remains incomplete. Some reports described that phosphorylated Relish is cleaved by caspase (Lan et al., 2013; Tassanakajon et al., 2018). Relish is able to move into the nucleus and activates the synthesis of AMPs, penaeidin (Tassanakajon et al., 2018). The JAK-STAT pathway may play crucial role in the response to Gram-negative bacteria and viral infection (Sun,

Shao, Zhang, Zhao, & Wang, 2011). JAK is downstream protein that associate with membrane receptor to initiate of transcription factor, STAT which acts downstream to regulate of the dynamin (Dnm) expression in shrimp (Li & Xiang, 2013b). The overview of downstream signal transductions of Toll, IMD, and JAK/STAT pathways in shrimp are presented in Figure 7.

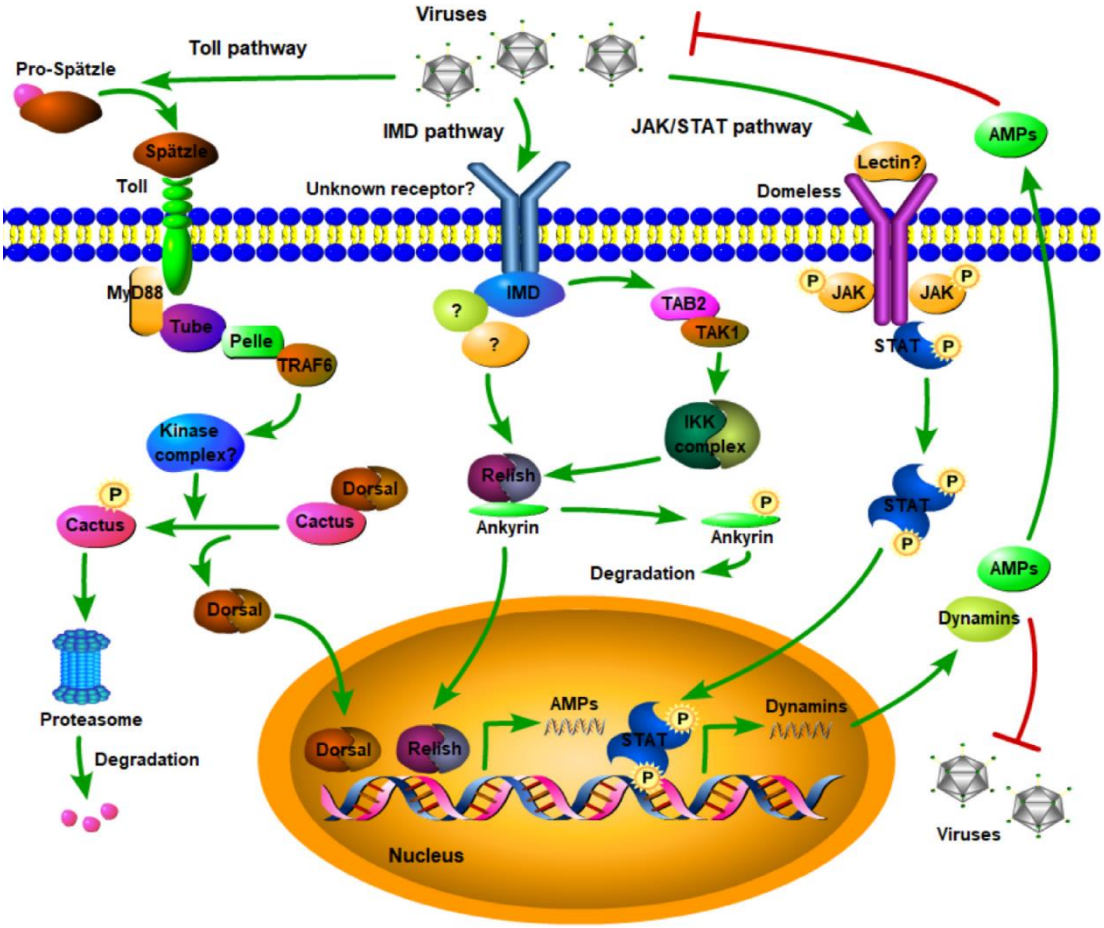



Figure 7 Toll, IMD, and JAK/STAT signaling pathways in *Macrobrachium*

Source: Huang, Y., & Ren, Q. (2021). Innate immune responses against viral pathogens in *Macrobrachium*. *Dev Comp Immunol, 117.*

| TNF Ligand Name | TNF Receptor Name |
|---|---|
| TNFSF1, LT-α | TNFR1, TNFR2 |
| TNFSF2, TNF-α | TNFR1/TNFR2, (TNFRSF 1A, DR1), TNFRSF1B |
| TNFSF3, LT-β | TNFRSF3, LT-βR |
| TNFSF4, OX40L, CD252, gp34 | TNFRSF4, OX40, CD134 |
| TNFSF5, CD40L, CD154, gp39 | TNFRSF5, CD40, p50 |
| TNFSF6, FasL, CD95L, Apo1L | TNFRSF6, Fas, CD95, Apo1, DR2 |
| TNFSF7, CD27L, CD70 | TNFRSF7 (CD27), TNFRSF6B (DcR3) |
| TNFSF8, CD30L, CD153 | TNFRSF8, CD30 |
| TNFSF9, 4-1BBL | TNFRSF9, 4-1BB, CD137, ILA |
| TNFSF10, TRAIL, Apo2L | TNFRSF10A (TRAILR1), DR4, Apo2<br>TNFRSF10B (TRAILR2), DR5<br>TNFRSF10C (TRAILR3), DcR1<br>TNFRSF10D (TRAILR4), DcR2 |
| TNFSF11, RANKL, TRANCE, OPGL, ODF | TNFRSF11A (RANK), TRANCER<br>TNFRSF11B (OPG), OCIF |
| TNFSF12, TWEAK, Apo3L | TNFRSF12A, Fn12, TWEAKR |
| TNFSF13, TALL-2, TRDL-1 | TNFRSF13A/17 (BCMA), TNFSF13B (TACI) |
| TNFSF13B, BLYS, THANK | TNFRSF13B (TACI), TNFRSF13C (BAFFR)<br>TNFRSF17 (BCMA) |
| TNFSF14, LIGHT, HVEML, LT-γ | TNFRSF14, LIGHTR, HVEM, LT-βR |
| TNFSF15, TL1A, VEGI | TNFRSF25 (DR3), DcR3 |
| TNFSF18, GITRL | TNFRSF18 (GITR), AITR |
| EDA-A1 | EDAR |
| EDA-A2 | TNFRSF27, XEDAR |
| NI | TNFRSF19, TROY, TAJ |
| NI | TNFRSF19L, RELT |
| Amyloid polypeptide (APP)**not a TNFSF member* | TNFRSF21, DR6 |
| NI | TNFRSF16 (NGFR), CD271 |

NI = No information

Figure 8. Members of the TNF superfamily (TNFSF)

Source: Modified from Dostert, C., Grusdat, M., Letellier, E., & Brenner, D. (2019). The TNF Family of Ligands and Receptors: Communication Modules in the Immune System and Beyond. *Physiological Reviews, 99*(1), 115-160.

### 3. Tumor necrosis factor

Tumor necrosis factor (TNF) is a critical cytokine that plays a crucial role in the cellular signaling pathways in the immune systems of vertebrates. TNF regulates various cellular functions such as cell proliferation, cell differentiation, which involves in cell apoptosis, cell inflammation, lymphocyte homeostasis, and tissue development (Ware, 2003). TNF also function as an effector molecule in host defense mechanisms by regulating cell survival and cell death. (Locksley, Killeen, & Lenardo, 2001). The TNF superfamily is currently known to be composed of 19 TNF ligands and 29 TNF receptors (TNFRs), as presented in Figure 8 (Dostert, Grusdat, Letellier, & Brenner, 2019). TNF is a type II transmembrane protein, which has an intracellular N-terminus and the extracellular C-terminus. The TNF homology domain (THD) is located in the C-terminus composed of 10 β-strands, forming as a compact like “jellyroll” topology, typical for TNF ligand superfamily (Wiens & Glenney, 2011). Two types of TNF ligands, membrane TNF (mTNF) and soluble TNF (sTNF) have an interaction with cognate membrane receptor, TNF receptor (TNFR), which have been indicated as TNFR1 and TNFR2 to bind with TNF ligand and initiate the TNF signal transduction for downstream signaling pathway in response to the pathogen infection (Wajant et al., 2003). The TNF signaling pathway can lead to activation of the NF-$\kappa$B and JNK pathway responsible for cell apoptosis and cell survival (Holbrook, Lara-Reyna, Jarosz-Griffiths, & McDermott, 2019). The mechanism of TNF signal transduction in vertebrate is described in Figure 9.

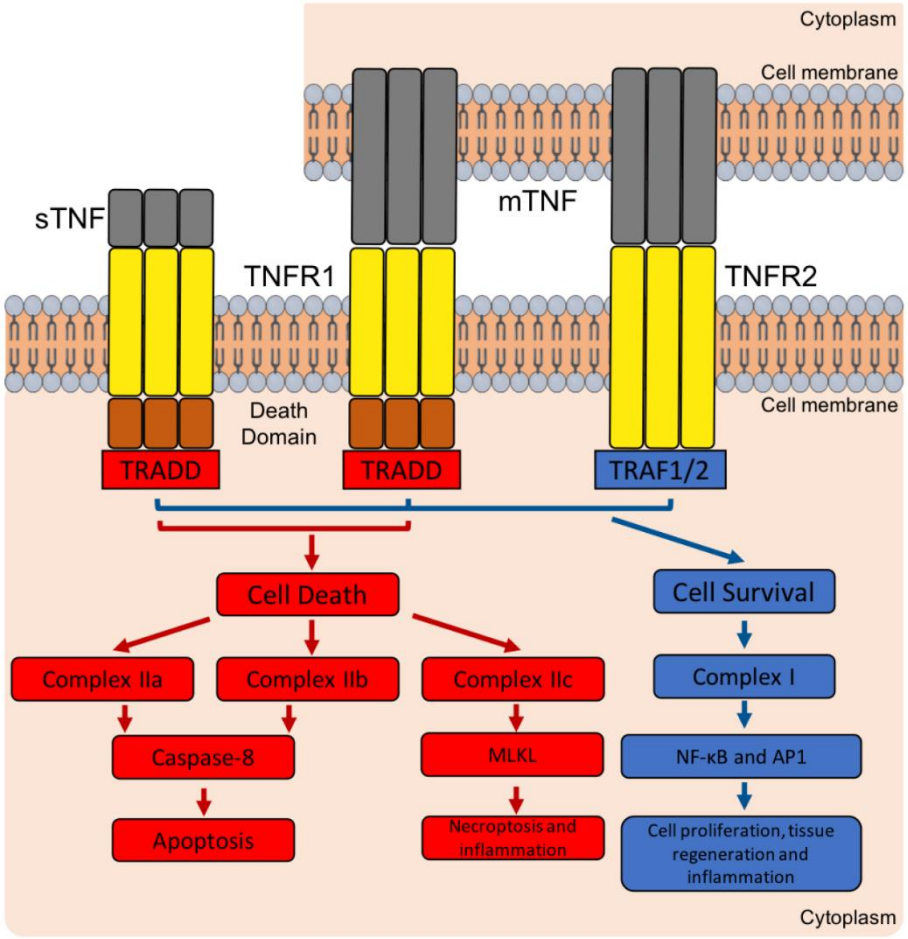



Figure 9 Overview of tumor necrosis factor (TNF) signaling pathways in vertebrates.

Source: Holbrook, J., Lara-Reyna, S., Jarosz-Griffiths, H., & McDermott, M. (2019). Tumour necrosis factor signalling in health and disease. *F1000Res, 8*

In the downstream of the TNF signaling pathway, TNF ligand binds to either TNFR1 or TNFR2. TNFR1 consists of death domain (DD) which interacts with the TNFR1-associated death domain (TRADD) to bind with several ligands, including receptor-interacting protein 1 (RIP1), TNFR-associated factor (TRAFs) such as TRAF2 and TRAF5. Upon recruitment, TRAF2 and TRAF5 associate with the cellular inhibitor of apoptosis protein 1 or 2 (cIAP1 or cIAP 2) to form a complex leading to ubiquitination of RIP1 and formation of the inhibitor of $\kappa$B kinase (I$\kappa$K) gamma (I$\kappa$K$\gamma$) complexes. The complex will trigger TAK1 complex, which activates IKK$\alpha$/$\beta$, JNK, and p38 signaling leading to activation of the NF-$\kappa$B and AP-1 pathway to synthesize the transcription factor or cytokine, chemokine to response against invading pathogen (Holbrook et al., 2019; Wajant et al., 2003). The TNFR2, which lacks the death domain, can directly associate with TRAF2 and TRAF5, forming a complex that activates the NF-$\kappa$B and AP-1 pathways. Moreover, TNF can activate cell apoptosis and cell necrosis. In TNFR1 signaling, TRADD recruits Fas-associated protein-containing death domain (FADD) which interacts with deubiquitinated RIP1 to cleave the pro-caspase-8 into caspase-8, initiating the caspase signaling cascade and leading to cell apoptosis. The ubiquitination of RIP1 can recruit the receptor-interacting protein 3 (RIP3) to form the RIP1-RIP3 necrosome complex which interacts with mixed lineage kinase domain-like protein (MLKL) and leads to activation of cell necrosis (Chu, 2013). The overview of TNF downstream signaling pathway in vertebrate is presented in Figure 10.

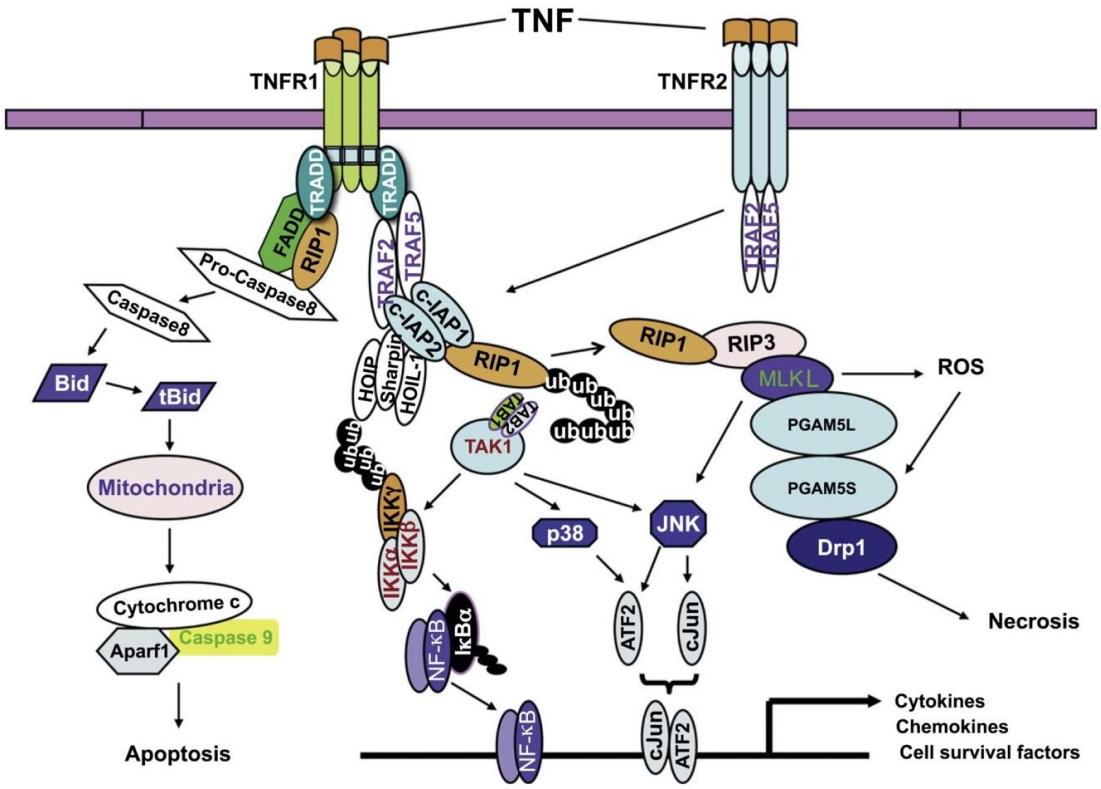



Figure 10 Downstream of the TNF signaling pathways in vertebrates.

Source: Wajant, H., Pfizenmaier, K., & Scheurich, P. (2003). Tumor necrosis factor signaling. *Cell Death & Differentiation, 10*(1), 45-65.

In invertebrates, the research of TNFSF members has not been reported to a great extent. The first characterization of TNF was identified in *Drosophila melanogaster* named as *Dm*Eiger and its recptor named as *Dm*Wengen. The identification of Eiger as a member of the TNFSF was completed by analyzing the ectodysplasin A2 (EDA-A2) homology domain of TNF through TBLASTN. The sequence analysis showed that *DmEiger* gene contains an open reading frame (ORF) of 1245 bp that encoded the deduced amino acid of 415 amino acids. *Dm*Eiger was indicated as type II membrane protein that consists of a cytoplasmic domain and a single transmembrane domain at N-terminus, and an extracellular domain at C-terminus. *Dm*Wengen was identified as TNFR using homology of the extracellular domain of human TNFR1. Wengen has been characterized as a type I or type III membrane protein that interact with Eiger and leads

to induce cell death indirectly by triggering JNK signaling pathway (Kanda, Igaki, Kanuka, Yagi, & Miura, 2002; Kauppila et al., 2003; Moreno et al., 2002). The first identification of TNFSF in mollusk was reported in disk abalone, *Haliotis discus* (*Ab*TNF-$\alpha$). The full length of *Ab*TNF-$\alpha$ consist of 930 bp encoding 239 amino acids which consisted of the signature transmembrane domain at N-terminus. The expression of *Ab*TNF-$\alpha$ was observed by *in vivo* experiment that stimulated with pathogenic bacteria, viral morrhagic septicaemia virus (VHSV), and lipopolysaccharide (LPS). *Ab*TNF-$\alpha$ showed up-regulation after inducing by bacteria, VHSV, and LPS. The results suggested that *Ab*TNF-$\alpha$ could respond to pathogenic infection and might play an important role in the innate immune system (De Zoysa, Jung, & Lee, 2009). In 2014, Sun and colleagues discovered TNFSF in Pacific oyster, *Crassostrea gigas* (*Cg*TNF-1). The mRNA expression level of *Cg*TNF-1 in hemocytes was significantly up-regulated after LPS stimulation. Co-stimulation of the recombinant *Cg*TNF-1 protein with LPS resulted in a significantly increased rate of apoptosis and phagocytosis in the haemocytes and increased the activities of phenoloxidase enzyme and lysozyme in the haemolymph. These results suggested that *Cg*TNF-1 plays a role in regulating the immune response by modulating processes such as hemocyte apoptosis and phagocytosis, antibacterial activity, and the activation of immune-related enzymes (Sun et al., 2014). Lately in 2020, Zheng and colleagues identified another TNFSF from *C. gigas* name as *Cg*TNF-2. The investigation of *Cg*TNF-2 expression showed that *Cg*TNF-2 was significantly up-regulated after stimulation by peptidoglycan (PGN) and LPS. The recombinant *Cg*TNF-2 protein was found to inhibit the growth of *Vibrio splendidus*. This finding suggest that *Cg*TNF-2 may played a role in stimulating the innate immune response of oyster by triggering the production of nitric oxide and inducing lysozyme activity, thereby enhancing the antibacterial activity (Zheng et al., 2020). Recently, TNFSF was reported in Chinese mitten crab, *Eriocheir sinesis* (*Es*TNFSF). The complete cDNA of *Es*TNFSF was 2462 bp consisted of ORF 1500 bp which encoding 499 amino acids. The deduced amino acid analysis revealed that *Es*TNFSF comprise of a transmembrane region, a low complex region, and a TNF domain. The qPCR analysis of *Es*TNFSF expression showed

that *Es*TNFSF expression level in hemocyte was up-regulated after stimulation with LPS, PGN, *S. aureus*, and *V. parahaemolyticus*. The results revealed that *Es*TNFSF may play a crucial role in response to antibacterial immune defense of *E. sinesis* (Huang, Si, Du, Du, & Ren, 2022).

**Tumor necrosis factor in shrimp**

The first identification of TNF in shrimp was reported in kuruma shrimp, *Marsupenaeus japonicus* (*MjTNF*) that consisted of an ORF 1868 bp encoding a predicted protein of 462 amino acids, containing a predicted transmembrane domain and the conserved TNF domain. *Mj*TNF homolog showed 30.7% and 26.7% identity with *Dm*Eiger and human ectodysplasin A, respectively. *Mj*TNF was found to be most highly expressed in muscle tissue, and *Mj*TNF expression was up-regulated in lymphoid organ cells after stimulating with *V. penaeicida*. The report suggested that *Mj*TNF might involve in the innate immunity by response to Gram-negative bacteria (Mekata et al., 2010).

Later in 2012, Wang and colleagues characterized TNF from *Litopenaeus vannamei* (*Lv*TNFSF), TNFRSF (*Lv*TNFRSF) and lipopolysaccharide-induced TNF-$\alpha$ factor (*Lv*LITAF). *Lv*TNFSF consisted of 472 amino acids and exhibited a conserved TNF domain at C-terminus, sharing 89.8% identity with *Mj*TNF. *Lv*TNFRSF was first identified in crustaceans which consisted of 296 amino acids with a single conserved TNFR domain and showed 89.8% with Farrer's scallop, *Chlamys farreri* TNFR, and 14.6% identity with *Drosophila* Wengen and human TNFR1. *Lv*LITAF was also the first reported in crustacean. *Lv*LITAF consisted of 124 amino acids with the LITAF domain and showed 62.6% and 32.3% identity with *Drosophila* LITAF and human LITAF, respectively. The report showed that *Lv*TNFSF, *Lv*TNFRSF and *Lv*LITAF could change after challenged with *S. aureus*, WSSV and *C. albicans*. Moreover, *Lv*TNFSF can be activated by *Lv*LITAF, *Lv*Relish, *Lv*Dorsal and *Lv*STAT which function together with LITAF, Toll, IMD, and JAK/STAT pathways in shrimp immune responses. It has been suggested that *Lv*TNFSF, and *Lv*TNFRSF may involve in shrimp immune responses to pathogenic infections (Wang et al., 2012).

In 2018, Gabina and colleagues reported TNF in Crayfish, *Procambarus clarkii* (*Pc*TNF) transcriptome using Next Generation Sequencing (NGS). *Pc*TNF consisted of 1524 bp which encoded 508 amino acids with conserved transmembrane domain and TNF domain. *Pc*TNF showed 48% identity with *Metanephrops japonicus* TNF. *Pc*TNF was identified as some elements of apoptosis components function as TNF-related apoptosis inducing ligand (TRAIL). (Calderón-Rosete, González-Barrios, Lara-Lozano, Piña-Leyva, & Rodríguez-Sosa, 2018)

Recently in 2019, Qin and colleagues reported TNF from *Macrobrachium nipponense* (*Mn*TNF), which consists of an ORF of 1407 bp encoding the deduced amino acid of 468 residues. *Mn*TNF was identified as type II membranes protein containing a transmembrane segment at N-terminus, a typical extracellular (THD) at C-terminus, and an extended extracellular portion. The TNF homology domain of *Mn*TNF showed identities of 76%, 38%, 27%, and 26% with *Mj*TNF, *Dm*Eiger, human TWEAK, and human EDA, respectively. The report showed that *Mn*TNF was significantly up-regulated upon *Aeromonas veronii* challenged. The *Mn*TNF silencing mediated RNAi led to up-regulation of AMPs, *crustin* and inhibited activation of proPO system. Moreover, after the bacteria challenge, there was a significant increase in the expression of the *relish gene,* which serves as the NF-$\kappa$B transcription factor gene. These findings indicate that *Mn*TNF potentially participates in immune response by regulating the expression of AMPs genes and the activity of phenoloxidase by coordination with the IMD pathway (Qin et al., 2019).

**4. Rapid Amplification of cDNA Ends (RACE)**

Rapid Amplification of cDNA Ends (RACE) is a widely used technique in molecular biology for obtaining the complete cDNA sequence. RACE, which relies on the polymerase chain reaction (PCR), allows for the amplification of a specific cDNA sequence starting from a small known sequence within the transcript towards either the 5' end (5'-RACE) or 3' end (3'-RACE) of the cDNA (Schaefer, 1995; Yeku & Frohman, 2011). The partial sequence of mRNA typically obtained using degenerate primers that are specifically designed based on conserved regions of interest gene.

To obtain the 5'-RACE, the first step involves using a gene-specific primer (GSP) along with reverse transcriptase to synthesize the first-strand cDNA from mRNA template. Subsequently, a homopolymeric tail is added to the 3' end of the first-strand cDNA through the action of terminal transferase. The second strand of cDNA is then synthesized through using a universal primer (UPM) to generate a double-stranded cDNA. The resulting 5'-RACE cDNA serves as the template for performing 5'-RACE PCR using both the UPM and GSP2. The UPM is designed specifically to target the homopolymeric tail. (Figure 11) (Chaivisuthangkura, 2018).

In the case of 3'-RACE, the initial step involves employing the oligo-dT-adaptor primer along with the mRNA template to synthesize the first-strand cDNA using reverse transcriptase. This is followed by elimination of the mRNA. Then, the second strand cDNA is then synthesized using GSP to generate a double-stranded cDNA. The resulting 3'-RACE cDNA is used as the template for performing 3'-RACE PCR with both the UPM and GSP2. The UPM is complementary sequence of the oligo-dT-adaptor primer (Figure 12). (Chaivisuthangkura, 2018).

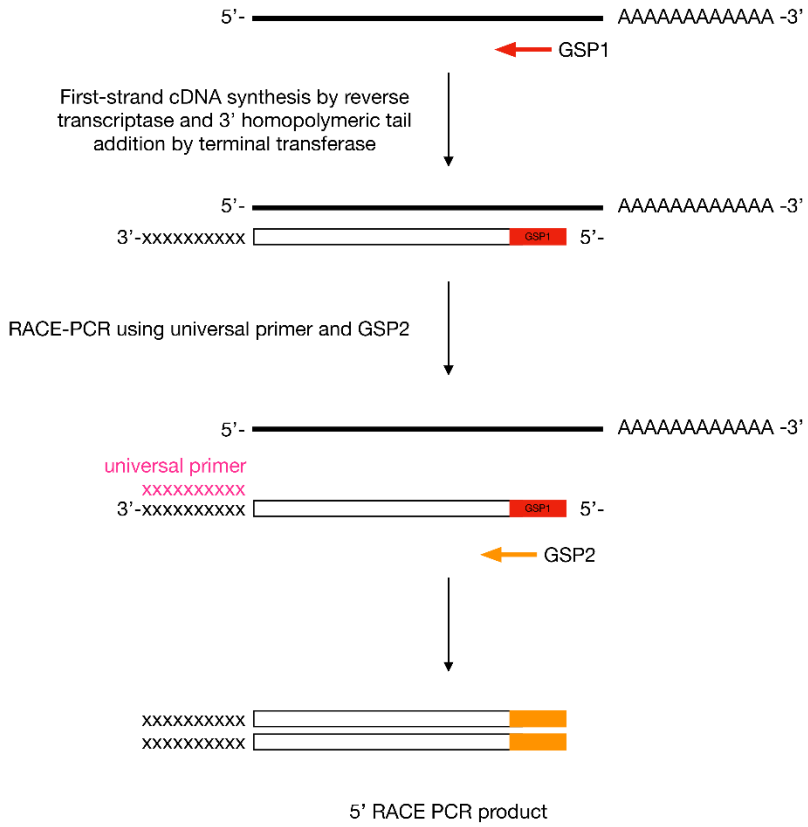



Figure 11 5'-RACE

Source: Modified from Chaivisuthangkura, P. (2018). *Genetic engineering and applications in research* (1st ed.). Bangkok, Thailand: Charansanitwong Printing Company Limited.

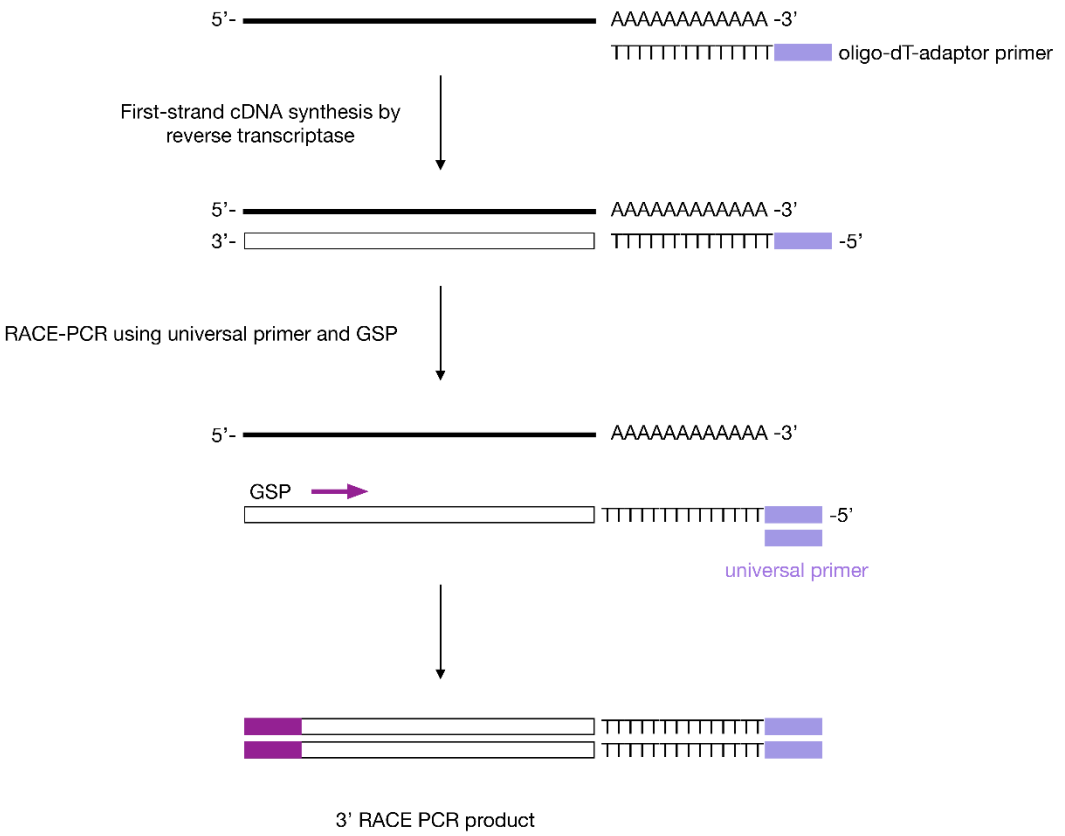



Figure 12 3'-RACE

Source: Modified from Chaivisuthangkura, P. (2018). *Genetic engineering and applications in research* (1st ed.). Bangkok, Thailand: Charansanitwong Printing Company Limited.

# CHAPTER 3
# MATERIALS AND METHODS

## Equipment and chemical reagent

### 1. Organism

1.1 *Macrobrachium rosenbergii*

1.2 *Escherichia coli* TOP 10

1.3 *Aeromonas hydrophila* VMARC1234

1.4 *Macrobrachium rosenbergii* nodavirus

### 2. Equipment

Equipment as listed in Table 1 were used in this study

Table 1 Equipment used in this study

| Equipment | Company |
|---|---|
| Thermal cycler | Bio-Rad, USA |
| Gel Doc™ XR+ | Bio-Rad, USA |
| Electrophoresis apparatus | Bio-Rad, USA |
| CFX Connect™ Real-Time PCR Detection System | Bio-Rad, USA |
| Refrigerated centrifuge | Eppendorf, Germany |
| Microcentrifuge 7M | Spectrafuge, South Korea |
| NanoDrop Lite Spectrophotometer | Thermo Fisher Scientific, USA |

### 3. Chemical reagent

Chemical reagent as listed in Table 2 were used in this study

Table 2 Chemical reagent used in this study

| Chemical reagent | Company |
|---|---|
| NucleoSpin® RNA Plus isolation Kit | MACHEREY-NAGEL, Germany |
| NucleoSpin® Gel and PCR Clean-up Kit | MACHEREY-NAGEL, Germany |
| NucleoSpin® Plasmid Kit | MACHEREY-NAGEL, Germany |
| NucleoSpin® Tissue Genomic DNA Purification Kit | MACHEREY-NAGEL, Germany |
| SMARTer® RACE cDNA Amplification kit | Clontech, USA |
| SeqAmp™ DNA Polymerase | Clontech, USA |
| Zero Blunt® TOPO® Cloning Kit | Invitrogen, USA |
| pCRTM8/GW/TOPO® TA Cloning® Kit | Invitrogen, USA |
| Platinum® *Taq* DNA Polymerase | Invitrogen, USA |
| SuperScript® III First-Strand Synthesis System for RT-PCR | Invitrogen, USA |
| SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* DNA Polymerase | Invitrogen, USA |
| KOD One™ PCR Master Mix | TOYOBO, Japan |
| SensiFAST™ cDNA Synthesis Kit | BioLine, USA |

**Methodology**

This research was processed in 5 major steps, as described in Figure 13. Firstly, molecular isolation and identification of the full-length cDNA of *MrTNF* by Rapid Amplification of cDNA End (RACE) was performed. Secondly, sequence analysis of the full-length cDNA of *MrTNF* was conducted using bioinformatic tools. Thirdly, tissue distribution and expression analysis were investigated using quantitative real-time RT-PCR (qRT-PCR). Fourthly, an immune challenge experiment with *A. hydrophila* was observed. Lastly, an immune challenge experiment with *Mr*NV was studied.

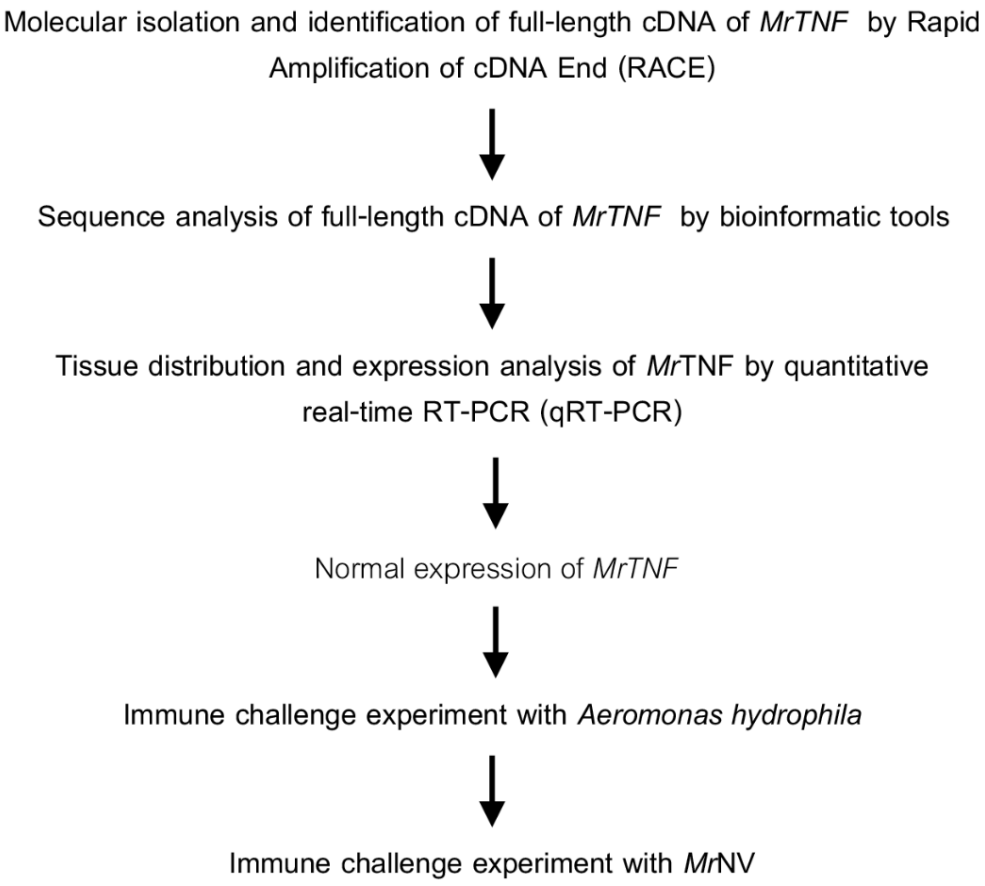



Figure 13 Research outline

**1. Molecular isolation and identification of full-length cDNA of *MrTNF* by Rapid Amplification of cDNA End (RACE)**

For molecular isolation of the full-length cDNA sequence of *MrTNF*, the partial sequence of *MrTNF* was obtained from Dr. Phongthana Phasookhush (Pasookhush et al., 2019). To collect the tissue sample from various organs for extract the total RNA and investigate the expression of *MrTNF*, the healthy *M. rosenbergii* were purchased from a local market in Bangkok, Thailand. In addition, for the immune challenge experiment, the sample of *M. rosenbergii* were obtained from a local farm at Bang Phra district, Chachoengsao province, Thailand.

**1.1 RNA extraction**

Total RNA was extracted from gills and hepatopancreas of *M. rosenbergii* (40-50 g body weight) using NucleoSpin® RNA Plus (MACHEREY-NAGEL, Germany) following the manufacturer's protocol. Tissue samples were homogenized with 350 µL of lysis buffer (LBP) and and vortexed before being briefly spun down. The lysate was filtrated using NucleoSpin® gDNA Removal Column by centrifugation at 11,000 x *g* for 30 seconds. Following this step, the flow-through supernatant was mixed with 100 µL of binding solution (BS) by pipetting up and down five times. To bind RNA, the mixed solution was transferred into NucleoSpin® RNA Plus column followed by centrifugation at 11,000 x *g* for 30 seconds. To wash and dry the silica membrane, 200 µL of buffer WB1 was added then centrifuged at 11,000 x *g* for 30 seconds. Following that, the column was washed with 600 µL of buffer WB2 then centrifuged at 11,000 x *g* for 30 seconds and washed with 250 µL of buffer WB2 and centrifuged at 11,000 x *g* for 2 minutes to dry the silica membrane. The total RNA was eluted twice with 30 µL of RNase-free water and centrifuged at 11,000 x *g* for 1 minute. The concentration of total RNA was measured using NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, USA). Total RNA was stored at -70°C until it was ready for use.

**1.2 The 5'- and 3'- RACE Ready cDNA synthesis**

The full-length of *MrTNF* was isolated using RACE, 5'- and 3'- RACE Ready cDNA was synthesized using the SMARTer® RACE cDNA Amplification kit (Clontech, USA) following the manufacturer's protocol. First, RACE Ready cDNA synthesis mixtures were prepared separately, as shown in Tables 3 and 4 for the 5'-RACE Ready cDNA synthesis and the 3'-RACE Ready cDNA synthesis, respectively.

Table 3 5'-RACE Ready cDNA synthesis mixture

| Component | Volume (µL) |
|---|---|
| RNA (1 µg/µL) | 1.0 |
| 5'-CDS Primer A | 1.0 |
| Sterile $H_2O$ | 9.0 |
| Total volume | 11.0 |

Table 4 3'-RACE Ready cDNA synthesis mixture

| Component | Volume (µL) |
|---|---|
| RNA (1 µg/µL) | 1.0 |
| 3'-CDS Primer A | 1.0 |
| Sterile $H_2O$ | 10.0 |
| Total volume | 12.0 |

Afterwards, the mixture for both 5'- and 3'-RACE Ready cDNA synthesis were incubated at 72°C for 3 minutes and 42°C for 2 minutes, followed by briefly spun down for 10 seconds. For the 5'-RACE Ready cDNA synthesis reaction, SMARTer II A Oligonucleotide was added with a volume of 1 µL, followed by mixing the cDNA synthesis mixture with reagents as shown in Table 5.

Table 5 5’ and 3’ -RACE Ready cDNA synthesis mixture

| Component | Volume (µL) |
|---|---|
| 5’ or 3’ -RACE cDNA synthesis mixture | 12.0 |
| 5X first strand buffer | 4.0 |
| SMARTScribe Reverse Transcriptase (100 U) | 2.0 |
| dNTPs (20 mM) | 1.0 |
| DTT (120 mM) | 0.5 |
| RNase inhibitor (40 U/µL) | 0.5 |
| Total volume | 20.0 |

To denature RNA, the 5’ and 3’-RACE Ready cDNA synthesis mixtures from Table 5 were incubated at 42°C for 90 minutes followed by incubated at 70°C for 10 minutes using a thermal cycler (Bio-Rad, USA). 5’- and 3’- RACE Ready cDNA was diluted with 90 µL of Tricine-EDTA buffer (RNA $\geq$ 200 ng) and stored at -20°C until use.

**1.3 Rapid amplification of cDNA ends (RACE)**

To construct the full-length cDNA of *MrTNF*, the RACE PCR reaction was performed by using SeqAmp™ DNA Polymerase (Clontech, USA). The primary PCR amplification was initially performed using RACE Ready cDNA from section 1.2 as a template, followed by the nested PCR which uses a diluted primary PCR product (2 µL of primary PCR products with 98 µL of Tricine-EDTA) as a template. The primary PCR and nested PCR amplification were performed with specific primers as shown in Table 6. The reaction mixtures of both primary and nested PCR were shown in Table 7 and Table 8, respectively.

Table 6 Primers used in this study

| Primer | Sequence |
|---|---|
| TNF.GSP001 (5'-GSP) | 5'-GGG CCT GAA GCT GGT GGG TAC CGC TGG-3' |
| TNF.NGSP001 (5'-NGSP) | 5'-CTC CGC CGC TCT TGT CGC TGT CGT CGC-3' |
| TNF.GSP002 (3'-GSP) | 5'-CTC CGC CGC TCT TGT CGC TGT CGT CGC-3' |
| TNF.NGSP002 (3'-NGSP) | 5'-CCT CAG CGG CCA TCG TGG TCC CAC AGA-3' |
| UPM | 5'-CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT-3' |
| UPM short | 5'-CTA ATA CGA CTC ACT ATA GGG C-3' |

Table 7 Primary PCR reaction mixture

| Component | Volume (µL) |
|---|---|
| 2X SeqAmp Buffer | 25.0 |
| PCR-Grade $H_2O$ | 15.5 |
| 10X UPM | 5.0 |
| 5' or 3'-RACE Ready cDNA | 2.5 |
| 5' or 3'- GSP (10 µM) | 1.0 |
| SeqAmp DNA Polymerase | 1.0 |
| Total volume | 50.0 |

The PCR reaction was performed using the thermal cycler (Bio-Rad, USA) under the condition as described below.

Primary PCR condition :

| | | | |
|---|---|---|---|
| Step 1 | Denaturation | 94°C | for 30 seconds |
| Step 2 | Annealing | 72°C | for 3 minutes |
| | Repeat steps 1 and 2 for 4 cycles | | |
| Step 3 | Denaturation | 94°C | for 30 seconds |
| Step 4 | Annealing | 70°C | for 30 seconds |
| Step 5 | Extension | 72°C | for 3 minutes |
| | Repeat steps 3, 4 and 5 for 4 cycles | | |

| | | | |
|---|---|---|---|
| Step 6 | Denaturation | 94°C | for 30 seconds |
| Step 7 | Annealing | 68°C | for 30 seconds |
| Step 8 | Extension | 72°C | for 3 minutes |
| | Repeat steps 6, 7 and 8 for 24 cycles | | |

Table 8 Nested PCR reaction mixture

| Component | Volume (µL) |
|---|---|
| 2X SeqAmp Buffer | 25.0 |
| PCR-Grade $H_2O$ | 17.0 |
| Diluted 5' or 3' Primary PCR product | 5.0 |
| 5' or 3'- NGSP (10 µM) | 1.0 |
| UPM short | 1.0 |
| SeqAmp DNA Polymerase | 1.0 |
| Total volume | 50.0 |

Nested PCR condition :

| | | | |
|---|---|---|---|
| Step 1 | Denaturation | 94°C | for 30 seconds |
| Step 2 | Annealing | 68°C | for 30 seconds |
| Step 3 | Extension | 72°C | for 3 minutes |
| | Repeat steps 1, 2 and 3 for 24 cycles | | |

PCR products were analyzed using 1% agarose gel electrophoresis and observed under the UV light with Gel Doc™ XR+ (Bio-Rad, USA). The anticipated size of the PCR product was purified using NucleoSpin® Gel and PCR Clean-up Kit (MACHEREY-NAGEL, Germany) following the manufacturer's protocol.

Briefly, 100 mg of PCR product in agarose gel was dissolved with 200 µL of Buffer NT1 and incubated at 50°C. The dissolved mixture was then transferred into a NucleoSpin® Gel and PCR clean-up column and centrifuged at 11,000 x *g* for 30 seconds. The column was washed with 700 µL of Buffer NT3 and centrifuged at 11,000 x *g* for 30 seconds. To dry the silica membrane, the column was centrifuged at 11,000 x

*g* for 1 minute. The product was then eluted with 15 µL of Buffer NE and centrifuged at 11,000 x *g* for 1 minute. Finally, the purified PCR product was stored at -20°C until use.

### 1.4 Molecular cloning and identification of *MrTNF* cDNA

The purified PCR products were cloned into pCR Blunt II-TOPO Vector using Zero Blunt® TOPO® Cloning Kit (Invitrogen, USA) as shown in Figure 14. The ligation mixture was prepared according to Table 9 and incubated for 30 minutes at 22°C. After that, the reaction was transformed into *E. coli* TOP 10 competent cells via heat-shock transformation.

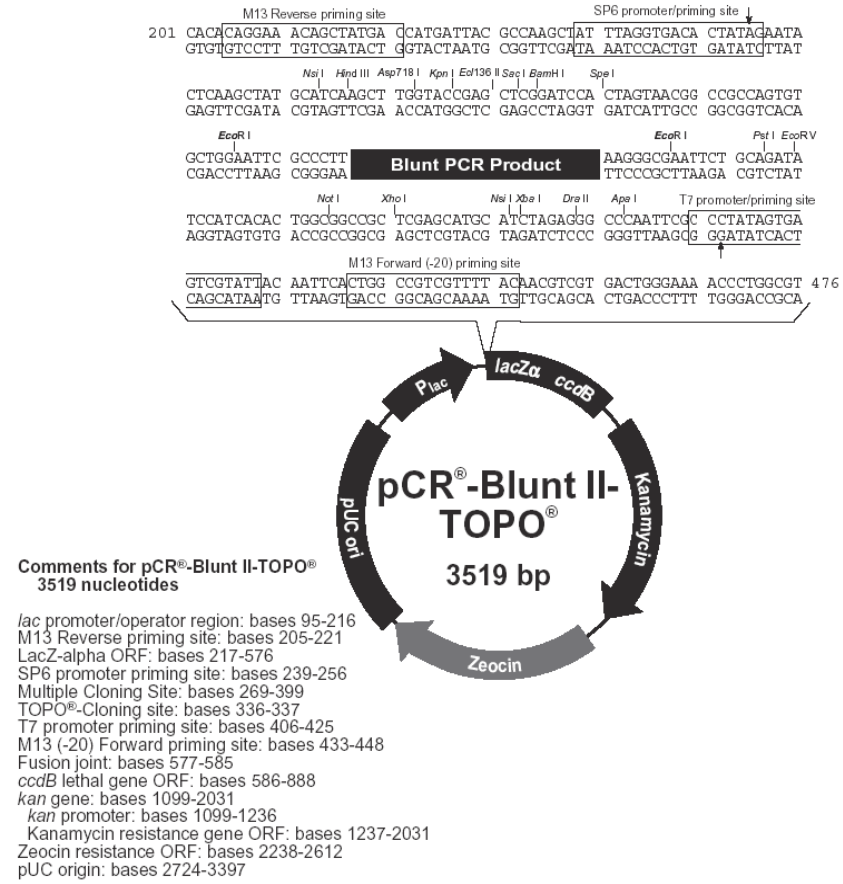



Figure 14 Map of pCR® Blunt II-TOPO® vector

Source: Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, USA)

Table 9 Ligation mixture for pCR-Blunt II-TOPO-*Mr*TNF

| Component | Volume (µL) |
|---|---|
| Purified *MrTNF* of nested PCR product | 4.0 |
| Salt solution | 1.0 |
| pCR Blunt II-TOPO Vector | 1.0 |
| Total volume | 6.0 |

After incubation, the ligation mixture was transformed into *E. coli* TOP 10 for sequencing. First, the ligation mixture was transferred to 200 µL of *E. coli* TOP10 competent cells and placed the tube on ice for 30 minutes. Subsequently, the bacterial cells were subjected to a 2-minute heat shock at 42°C, followed by immediate placement on ice for 20 minutes. After that, the mixture was added with 600 µL of LB and incubate at 37°C for 2 hours with shaking at 225 rpm. To increase the concentration of transformed competent cells, the mixture of transformed *E. coli* was subjected to centrifugation at 2,000 x *g* for 3 minutes. After that, 500 µL of supernatant was removed, and the remaining lysate was mixed by pipetting up and down five times. Finally, 100 µL of transformed *E. coli* cells were spread on LB agar plate containing 100 µg/mL of kanamycin, 40 µg/mL of X-gal, and 50 µg/mL of IPTG. Then, the LB agar plate was subsequently incubated overnight at 37°C.

The transformed E. coli cells carrying the MrTNF recombinant plasmid were observed as white colonies on the LB agar plate, which had been supplemented with kanamycin, X-gal, and IPTG. These white colony of *MrTNF* recombinant plasmid were transferred to 5 mL of LB broth containing kanamycin at final concentration of 100 µg/mL. The culture was then incubated at 37°C with shaking at 225 rpm for 16 hours.

The *MrTNF* recombinant plasmid was extracted from the *E. coli* using NucleoSpin® Plasmid Kit (MACHEREY-NAGEL, Germany) following the manufacturer's protocol. Firstly, bacterial cells were harvested by transferring the culture into sterile 1.5 mL tube and then centrifuged at 11,000 x *g* for 30 seconds. To lyse bacterial cells, the pellet was resuspended by vortexing with 150 µL of A1 buffer. The resuspended cell was added with 250 µL of A2 buffer and mixed by inverted tube for 5 times, followed by

incubation at room temperature for 2 minutes. Next, the supernatant was added with 350 µL of A3 buffer followed by inverting tube until the lysate turns colorless. Afterwards, the pellet was obtained by centrifugation at 12,000 x *g* for 3 minutes. To bind DNA, a NucleoSpin® Plasmid Easy Pure column was placed into the collection tube, and the supernatant was loaded into the column followed by centrifugation at 2,000 x *g* for 30 seconds. The column membrane was washed with 450 µL of AQ buffer and centrifuged at 12,000 x *g* for 1 minute. To elute the Plasmid DNA, the column was added with 50 µL of AE buffer then incubated for 1 minute at room temperature and centrifuged at 12,000 x *g* for 1 minute. The extracted recombinant plasmid was stored at -20°C until use.

To confirm the *MrTNF* recombinant plasmid, the extracted plasmid was digested with *EcoRI*-HF restriction enzyme (New England Biolabs, UK) following the manufacturer's protocol. The digestion mixture was prepared as shown in Table 10 then incubated for 2 hours at 37°C. Afterward, the products of digestion were analyzed by 1% agarose gel electrophoresis and observed under UV light using Gel Doc™ XR+ (Bio-Rad, USA).

Table 10 Digestion mixture for pCR-Blunt II-TOPO-*MrTNF*

| Component | Volume (µL) |
|---|---|
| Purified plasmid pCR Blunt II-TOPO-*MrTNF* | 8.0 |
| 10X CutSmart Buffer | 1.0 |
| *EcoRI*-HF (20,000 U/mL) | 1.0 |
| Total volume | 10.0 |

### 1.5 Molecular re-identification of *MrTNF*

To identify of 5'-end and 3'-end *MrTNF* sequence, the purified plasmid was sequenced by Sanger's sequencing (1st base laboratory, Malaysia). The site of restriction enzyme in plasmid regions was eliminated and the data was analyzed using BLASTx program from NCBI (https://blast.ncbi.nlm.nih.gov/blast/Blast.cgi). After alignment, the 5'-end and 3'-end sequences of *MrTNF* were merged to obtain the full-length cDNA of *MrTNF*.

### 1.5.1 The first strand cDNA synthesis

The first strand cDNA of *MrTNF* was synthesized from the total RNA in section 1.1 using SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, USA) following the manufacturer's protocol. The RNA mixture was prepared as shown in Table 11 and then incubated for 5 minutes at 65°C before being cooled on ice for 1 minute.

Table 11 RNA mixture

| Component | Volume (µL) |
|---|---|
| RNA | 8.0 |
| 50 mM Oligo(dT) | 1.0 |
| 10 mM dNTPs | 1.0 |
| Total volume | 10.0 |

Following the synthesis of the first-strand cDNA of *MrTNF*, a 2X reaction mixture was prepared according to the components listed in Table 12. Subsequently, the reaction mixture was subjected to incubation at 50°C for 50 minutes, followed by incubation at 85°C for 5 minutes using a thermal cycler (Bio-Rad, USA). To terminate the reaction, the mixture was cooled for 1 minute on ice, and 1 µL of *E. coli* RNase H (20 U/µL) was added. Finally, the first strand cDNA was incubated for 20 minutes at 37°C and stored at -20°C for later use.

Table 12 2X reaction mixture for the first-strand cDNA synthesis

| Component | Volume (µL) |
|---|---|
| RNA mixture (Table 11) | 10.0 |
| 25 mM $MgCl_2$ | 4.0 |
| 10X RT Buffer | 2.0 |
| 0.1 M DTT | 2.0 |

Table 12 (Continued)

| Component | Volume (μL) |
|---|---|
| RNaseOUT™ (400 U/μL) | 1.0 |
| SuperScript® III RT (200 U/μL) | 1.0 |
| Total | 20.0 |

### 1.5.2 Verification of full-length cDNA of *MrTNF* by PCR

To confirm the full-length cDNA of *Mr*TNF, the specific primers were designed from the start and stop codons of the *Mr*TNF sequence, which covered the entire coding sequence (CDS). The primer sequences were listed in Table 13.

Table 13 Primers for *MrTNF* verification

| Primer | Sequence |
|---|---|
| TNF.CDS_F | 5'-GGA GTG AAC GCC CTC CCT TGT A-3' |
| TNF.CDS_R | 5'-GAA GCG CTC AGG TAC CGC TTG TAG-3' |

The PCR reaction was performed using KOD One™ PCR Master Mix (TOYOBO, Japan) following the manufacturer's protocol. The reaction mixture was prepared as shown in Table 14. The cDNA from section 1.5.1 was used as DNA template.

Table 14 KOD One™ PCR reaction mixture

| Component | Volume (μL) | Final concentration |
|---|---|---|
| KOD One™ master mixture | 12.5 | 1X |
| $dH_2O$ | 5.5 | |
| cDNA (Template) | 5.0 | |
| 10 μM TNF.CDS_F | 1.0 | 0.3 μM |
| 10 μM TNF.CDS_R | 1.0 | 0.3 μM |
| Total volume | 25.0 | |

Finally, the PCR reaction was transferred into the thermal cycler (Bio-Rad, USA) under condition as described below.

PCR condition:

| | | | |
|---|---|---|---|
| Step 1 | Denaturation | 98°C | for 30 seconds |
| Step 2 | Annealing | 56°C | for 30 seconds |
| Step 3 | Extension | 68°C | for 30 seconds |

Repeat steps 1, 2 and 3 for 29 cycles

PCR products were analyzed by 1% agarose gel electrophoresis and observed under the UV light using Gel Doc™ XR+ (Bio-Rad, USA). The expected band was purified using NucleoSpin® Gel and PCR Clean-up Kit (MACHEREY-NAGEL, Germany) following the manufacturer's protocol as described in section 1.3.

### 1.5.3 Cloning of full-length cDNA of *MrTNF*

The purified PCR products of *MrTNF* coding sequence from section 1.5.2 were successfully inserted into the pCR Blunt II-TOPO vector with Zero Blunt® TOPO® Cloning Kit (Invitrogen, USA). The ligation mixture was prepared as listed in Table 15. Firstly, Subsequently, the reaction mixture was subjected to heat-shock transformation to introduce the plasmid into *E. coli* TOP10 competent cells. Afterward, the transformed *E. coli* was spread on LB-agar plate, as described in section 1.4.

Table 15 Ligation mixture for pCR-Blunt II-TOPO-*Mr*TNF CDS

| Component | Volume (µL) |
|---|---|
| Purified PCR product of *Mr*TNF CDS | 4.0 |
| Salt solution | 1.0 |
| pCR Blunt II-TOPO Vector | 1.0 |
| Total volume | 6.0 |

To confirm the recombinant plasmid *MrTNF* coding sequence, the extracted plasmid was digested with *EcoRI*-HF restriction enzyme (New England Biolabs, UK) following the manufacturer's protocol. The digestion mixture was prepared

as shown in Table 16 and then incubated for 2 hours at 37°C. After that, the product was analyzed by 1% agarose gel electrophoresis and observed under the UV light using Gel Doc™ XR+ (Bio-Rad, USA).

Table 16 Digestion mixture for pCR-Blunt II-TOPO-*MrTNF* CDS

| Component | Volume (µL) |
|---|---|
| Purified plasmid pCR Blunt II-TOPO-*Mr*TNF CDS | 8.0 |
| 10X CutSmart Buffer | 1.0 |
| *EcoRI*-HF (20,000 U/mL) | 1.0 |
| Total volume | 10.0 |

The extracted *MrTNF* coding sequence plasmid was further sequenced using Sanger's sequencing (1st base laboratory, Malaysia). The sequencing data was subjected to analysis using BLASTx program (https://blast.ncbi.nlm.nih.gov/blast/Blast.cgi). After identifying the coding sequence of *MrTNF*, the plasmid regions were eliminated, and the sequence was aligned and compared with the *MrTNF* cDNA sequence from section 1.4. to confirm and obtain the full-length *MrTNF* cDNA.

**2. Sequence analysis of full-length cDNA of *MrTNF* by bioinformatic tools**

**2.1 Protein analysis of *Mr*TNF**

To obtain the deduced amino acid of *Mr*TNF, the full-length *MrTNF* cDNA was translated into *Mr*TNF protein using ORF Finder program (https://www.ncbi.-nlm.nih.gov/orffinder/). The molecular weight and isoelectric point of *Mr*TNF protein were determined using Compute pI/Mw tool (https://web.expasy.org/ compute_pi/). The signal peptide and structural domains of *Mr*TNF was predicted using the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). To compare *Mr*TNF proteins with TNF of other species, the pairwise alignment was analyzed using Ident and Sim webserver (https://www.bioinformatics.org/sms2/ident_sim.html). In addition, the multiple sequence alignment was analyzed using MUSCLE tool (https://www.ebi.ac.uk/Tools/msa/muscle/).

### 2.2 Phylogenetic tree analysis

To study the relationship between *Mr*TNF and other TNFs in crustaceans, invertebrates, and vertebrates, the phylogenetic tree was constructed by Molecular Evolutionary Genetics Analysis (MEGA) software version X (http://www.megasoftware.net/). The amino acid sequences of the conserved TNF homology domain (THD) of *Mr*TNF and TNFs from other species were utilized in the construction of the phylogenetic tree using the maximum likelihood algorithm with Jones-Taylor-Thornton (JTT) matrix-based model and 1000 replicates of bootstrap analysis.

## 3. Tissue distribution and expression analysis of *MrTNF* by quantitative real-time RT-PCR (qRT-PCR)

### 3.1 Tissue collection and RNA extraction

Tissues of healthy *M. rosenbergii* (45-50 g body weight) were collected from several organs containing gill, hepatopancreas, heart, stomach, intestine, muscle and hemocyte. To collect the hemocyte, hemolymph was collected from ventral sinus using a 1 mL syringe and mixed with an equal volume of anticoagulant (Alsever's solution; 0.055% citric acid, 0.8% sodium citrate, 2.05% D-glucose, and 0.42% sodium chloride (w/v)). Afterward, hemocytes were collected by centrifugation at 1,500 x *g* for 10 minutes at 4°C. Then, the cell pellet was washed twice with 1 mL of Alsever's solution then centrifuged at 1,500 x *g* for 5 minutes at 4°C. Finally, the pellet of hemocyte was collected using centrifuged at 1,500 x *g* for 5 minutes at 4°C. Tissue organs and hemocytes pellet were stored at -70°C until use.

The extraction of total RNA from the collected tissues was performed using the NucleoSpin® RNA Plus Kit (MACHEREY-NAGEL, Germany) following the manufacturer's protocol, as described in section 1.1. The concentration of extracted total RNA was measured using NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, USA). Total RNA was stored at -70°C until use.

**3.2 cDNA synthesis for quantitative real-time RT-PCR (qRT-PCR)**

The total of RNA from section 3.1 was used as a template for cDNA synthesis using SensiFAST™ cDNA Synthesis Kit (BioLine, USA) following the manufacturer's protocol. In summary, a cDNA synthesis reaction mixture was prepared as shown in Table 17.

Table 17 SensiFAST™ cDNA synthesis reaction mixture

| Component | Volume (µL) |
|---|---|
| Total RNA (up to 1 µg) | n |
| 5X TransAmp Buffer | 4.0 |
| Reverse Transcriptase | 1.0 |
| $dH_2O$ | up to 20 |
| Total volume | 20.0 |

The qRT-PCR reaction was performed using the thermal cycler (Bio-Rad, USA) with condition as described below

| | | | |
|---|---|---|---|
| Step 1 | Primer annealing | 25°C | for 10 minutes |
| Step 2 | Reverse transcription | 42°C | for 15 minutes |
| Step 3 | Inactivation | 85°C | for 5 minutes |
| Step 4 | Hold | 4°C | |

Finally, cDNA was diluted into 500 ng/µL for qPCR reaction. The concentration of cDNA was measured using NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, USA). Then, cDNA was stored at -20°C until use.

**3.3 Normal expression analysis of *MrTNF* by quantitative real-time RT-PCR (qRT-PCR)**

The cDNA of each tissue from section 3.2 was used as a DNA template for studying the tissue distribution of *MrTNF*. The primers were designed from the partial

sequences of *MrTNF* using Primer3Plus tools (https://www.bioinformatics.nl/cgibin/-primer3plus/primer3plus.cgi) then elongation factor 1 alpha (EF1$\alpha$) gene was used as an internal control. The primers used for tissue distribution as listed in Table 18.

Table 18 PCR primers used for tissue distribution of *MrTNF*

| Primer | Sequence |
|---|---|
| TNF.qPCR_F6 | 5'-ATC ACC CTG GGA CAT TTC G-3' |
| TNF.qPCR_R6 | 5'-TCC CAG ATT GTC CAT CCA AG-3' |
| EF1$\alpha$ _F | 5'-TGC GCT GTG TTG ATT GTA GC-3' |
| EF1$\alpha$ _R | 5'-ACA ATG AGC TGC TTG ACA CC-3' |

The qRT-PCR reaction was performed using SensiFAST™ SYBR® No-ROX Kit (BioLine, USA) following the manufacturer's protocol. The qRT-PCR mixture was prepared as shown in Table 19.

Table 19 SensiFAST™ qRT-PCR reaction mixture

| Component | Volume (µL) | Final concentration |
|---|---|---|
| 2X SensiFAST SYBR® No-ROX Mix | 10.0 | 1X |
| 10 µM Forward primer | 0.8 | 0.4 µM |
| 10 µM Reverse primer | 0.8 | 0.4 µM |
| 500 ng/µL of cDNA from section 3.2 | 2.0 | |
| $dH_2O$ | 6.4 | |
| Total volume | 20.0 | |

The qRT-PCR was carried out using CFX Connect™ Real-Time PCR Detection System (Bio-Rad, USA) following qPCR conditions as described below.

qPCR condition :

| | | | |
|---|---|---|---|
| Step 1 | Polymerase activation | 95°C | for 2 minutes |
| Step 2 | Denaturation | 95°C | for 5 seconds |
| Step 3 | Annealing | 60°C | for 10 seconds |

| | | | |
|---|---|---|---|
| Step 4 | Extension | 72°C | for 10 seconds |
| | Plate read, repeat steps 2-4 for 39 cycles | | |
| Step 5 | Melt curve analysis | 65°C to 95°C | increment 0.5°C |
| | Plate read | | |

All experiments were performed in triplicate. The relative expression level of the *MrTNF* was analyzed by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) and the expression value was evaluated by corresponding to the n-fold difference relative to the control. The results were expressed as mean ± SD (standard deviation). The expression between time-point was analyzed by One-way analysis of variance (ANOVA) with Post Hoc Tukey test. Statistically significant differences were identified at $p<0.05$.

**4. Immune challenge experiment with *A. hydrophila***

To investigate the role of *Mr*TNF in the immune system, a Gram-negative bateria, *A. hydrophila* was injected into the healthy prawn. The expression of *MrTNF* in selected tissues were examined using qRT-PCR. The expression in infected prawns was compared with control group to determine the response of *Mr*TNF against bacterial infection.

**4.1 Confirmation and preparation of *A. hydrophila***

*A. hydrophila* (VMARC1234) were spread on tryptone soy agar (TSA) plate and incubated at 37°C for 16 hours. After that, a single white colony was selected and cultured with 4 µL of tryptone soy broth (TSB) at 37°C for 16 hours. Genomic DNA from *A. hydrophila* culture was then extracted using NucleoSpin® Tissue Genomic DNA Purification Kit (MACHEREY-NAGEL, Germany) following the manufacturer's protocol. Firstly, bacterial culture was centrifuged at 8,000 x *g* for 5 minutes, and the supernatant was carefully removed. Next, the pellet was resuspended with 180 µL of T1 buffer and mixed by pipetting up and down for 5 time Subsequently, 25 µL of proteinase K was added to the suspension, briefly vortexed, and incubated at 56°C for 2 hours (vortexing occasionally during incubation). Afterward, the bacterial cells were lysed with 210 µL of absolute ethanol and mixed up by vortexing briefly. To bind DNA, the lysate was filtered

through NucleoSpin® Tissue Column and centrifuged at 11,000 x *g* for 1 minute, the flow-through was discarded. The silica membrane of the column was washed with 500 µL of buffer BW and centrifuged at 11,000 x *g* for 1 minute. Subsequently, 600 µL of B5 buffer was added and centrifuged at 11,000 x *g* for 1 minute. To dry the silica membrane, the column was centrifuged at 11,000 x *g* for 1 minute. Finally, the bacterial DNA was eluted twice with 50 µL of prewarmed BE buffer (70°C) and incubated at room temperature for 1 minute followed by centrifugation at 11,000 x *g* for 1 minute. The bacterial DNA was stored at -20°C until use.

To confirm the *A. hydrophila* (VMARC1234) strain, PCR amplification was conducted using Platinum® *Taq* DNA Polymerase (Invitrogen, USA) along with specific primers (Wang et al., 2003) listed in Table 20. The reaction mixture was prepared as shown in Table 21.

Table 20 Primers used for *A. hydrophila* confirmation

| Primer | Sequence |
|---|---|
| AHH1F | 5'- GCC GAG CGC CCA GAA GGT GAG TT-3' |
| AHH1R | 5'- GAG CGG CTG GAT GCG GTT GT -3' |

Table 21 PCR reaction mixture

| Component | Volume (µL) | Final concentration |
|---|---|---|
| 10X PCR Buffer (-$Mg^{2+}$) | 5.0 | 1X |
| 50 mM $MgCl_2$ | 1.5 | 1.5 µM |
| 10 mM dNTPs | 1.0 | 0.2 µM |
| 10 µM forward primer | 1.0 | 0.2 µM |
| 10 µM reverse primer | 1.0 | 0.2 µM |
| Template DNA (*A. hydorophila*) | 1.0 | |
| Platinum® *Taq* DNA Polymerase (5U/µL) | 0.2 | 1U |
| $dH_2O$ | 39.3 | |
| Total volume | 50.0 | |

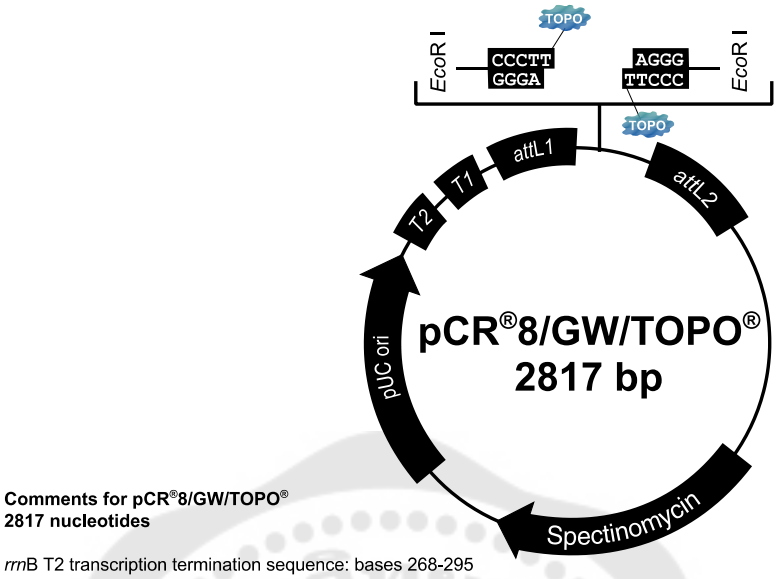
The PCR reaction tube was placed into the thermal cycler (Bio-Rad, USA) under condition as described below.

PCR condition :

| | | | |
|---|---|---|---|
| Step 1 | Initial denaturation | 95°C | for 2 minutes |
| Step 2 | Denaturation | 95°C | for 5 seconds |
| Step 3 | Annealing | 60°C | for 10 seconds |
| Step 4 | Extension | 72°C | for 10 seconds |

Repeat steps 2-4 for 29 cycles

The PCR products were subjected to electrophoresis on a 1% agarose gel and the product with expected size was purified using the method outlined in section 1.3. The purified PCR products of *A. hydrophila* was cloned into pCR™8/GW/TOPO® TA vector (Figure 15) using pCRTM8/GW/TOPO® TA Cloning® Kit (Invitrogen, USA). The ligation mixture for this process was prepared as shown in Table 22. Subsequently,the ligation mixture was transformed into *E. coli* TOP10 competent cells using the heat-shock transformation. Afterward, transformed *E. coli* was spread on TSA plate and was incubated to select the single colony for the plasmid extraction as described in section 1.4.




**Comments for pCR®8/GW/TOPO®**
**2817 nucleotides**

*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
*att*L1: bases 569-668
GW1 priming site: bases 607-631
TOPO® recognition site 1: bases 678-682
TOPO® recognition site 2: bases 683-687
*att*L2: bases 696-795
GW2 priming site: bases 733-757
T7 Promoter/priming site: 812-831 (c)
M13 reverse priming site: bases 836-852
Spectinomycin promoter: bases 930-1063
Spectinomycin resistance gene (Spn$^R$): 1064-2074
pUC origin: bases 2141-2814

(c) = complementary sequence

Figure 15 Map of pCR™8/GW/TOPO® TA vector

Source: pCR™8/GW/TOPO® TA Cloning® Kit (Invitrogen, USA)

Table 22 Ligation mixture for pCR™8/GW/TOPO® TA *A. hydrophila* PCR product

| Component | Volume (µL) |
|---|---|
| Purified *A. hydrophila* PCR product | 4.0 |
| Salt solution | 1.0 |
| pCR™8/GW/TOPO® TA | 1.0 |
| Total volume | 6.0 |

To investigate the recombinant plasmid containing the *hemolysin* gene of *A. hydrophila*, the extracted plasmid was digested using *EcoRI*-HF restriction enzyme (New England Biolabs, England) following the manufacturer's protocol. The digestion mixture was prepared as shown in Table 23 and incubated at 37°C for 2 hours. Subsequently, the resulting products were analyzed by1% agarose gel electrophoresis and observed under the UV light using Gel Doc™ XR+ (Bio-Rad, USA). The extracted *A. hydrophila* recombinant plasmid was further sequenced to confirm bacterial strain using Sanger's sequencing (1st base laboratory, Malaysia). The sequencing data was analyzed using BLASTn program (https://blast.ncbi.nlm.nih.gov/blast/Blast.cgi).

Table 23 Digestion mixture for pCR™8/GW/TOPO® TA *A. hydrophila* PCR product

| Component | Volume (µL) |
|---|---|
| Purified plasmid pCR™8/GW/TOPO® TA *A. hydrophila* | 8.0 |
| 10X CutSmart Buffer | 1.0 |
| *EcoRI*-HF (20,000 U/mL) | 1.0 |
| Total volume | 10.0 |

To prepare *A. hydrophila* inoculum, the bacteria were cultured in 4 mL of TSB and incubated with shaking at 37°C for overnight. The bacterial culture were sub-cultured in 20 mL of TSB and incubated with shaking at 37°C for 3 hours. The optical density (OD) was measured at wavelength of 600 nm. Afterward, bacterial culture were collected when OD600 reaches 0.5-0.7. Then, the bacterial cells were harvested by centrifugation at 2,000 x *g* for 20 minutes and the pellet were resuspended with 15 mL of sterile 2X PBS. After that, the bacterial cells were adjusted to an OD600 of 1. Therefore, bacterial cells were attained to $1x10^9$ CFU/mL. Bacterial cells suspension was diluted to $2x10^3$ CFU/µL for injection in the *A. hydrophila* challenged experiment.

### 4.2 *A. hydrophila* challenged experiment

The healthy prawns (10-15 g body weight) were divided into 2 groups. The first group, *A. hydrophila* challenged prawns and the second group, 2X PBS challenged

prawns as a control group. In *A. hydrophila* challenged group, each prawn was injected with 100 µL of *A. hydrophila* ($2x10^3$ CFU/µL) as described in previous study (Vaniksampanna et al., 2019). In control group, each prawn was injected with 100 µL of sterile 2X PBS. Next, the target organs from each group were collected at 0, 3, 6, 12, 24, 36 and 48 hours post injection (hpi). Total RNA from collected target organs was extracted using NucleoSpin® RNA Plus (MACHEREY-NAGEL, Germany) following the manufacturer's protocol, as outlined insection 1.1. The expression levels of *MrTNF* in target organs after challenged with *A. hydrophila* were performed using qPCR as described in section 3.3. The qRT-PCR analysis was conducted in triplicate, and the results were presented as mean ± SD. To determine differences among the experiments, the expression between challenge group and control group was analyze by Student's *t* test and One-way analysis of variance (ANOVA) with Post Hoc Tukey test. Statistically significant differences were identified at $p<0.05$.

To confirm the infection of *A. hydrophila* challenged prawns, the *A. hydrophila* injected prawns were randomly selected. Each selected prawn was sampled for 10 µL of hemolymph and mixed up with 90 µL of sterile 2X PBS. The mixture was diluted to $10^{-3}$ and $10^{-4}$ fold dilutions. An aliquot of 100 µL of each dilution was spread onto TSA plate and incubated plate at 37°C for overnight. The single bacterial colony was picked for testing the PCR amplification using Platinum® *Taq* DNA Polymerase (Invitrogen, USA) as described in section 3.4.1.

**5. Immune challenge experiment with *Mr*NV**

To study the role of *MrTNF* in immune response via viral infection, The experiment was challenged with *Mr*NV. The expression level of *MrTNF* infected prawns was compared with control group to investigate the response of *Mr*TNF against viral infection.

**5.1 Confirmation of *Mr*NV**

The sample of naturally *Mr*NV infected prawns (1-2 g body weight) were random collected from local farm in Song Phi Nong district, Suphan Buri province, Thailand. To confirm *Mr*NV infection, the viral nucleic acid was extracted from muscle of

*M. rosenbergii* prawn using High Pure Viral Nucleic Acid Kit (Roche, Switzerland) following the manufacturer's protocol. Firstly, the tissue sample was homogenized with 200 µL of lysis buffer then added with working solution (200 µL of binding buffer, 4 µL of poly (A) solution and 50 µL of proteinase K) and incubated the mixture at 72°C for 10 minutes. Following the incubation, 100 µL of isopropanol was added to the mixture then mixed by inverting tube up and down for 4-5 times. The lysate was filtrated using High Pure Filter by centrifugation at 8,000 x *g* for 1 minute. After that, the flow through was discarded and added 500 µL of inhibitor removal buffer followed by centrifugation at 8,000 x *g* for 1 minute. Afterward, the silica membrane was washed twice with 450 µL of wash buffer then centrifuged at 8,000 x *g* for 1 minute. Then, the silica membrane was dried by centrifugation at 13,000 x *g* for 1 minute. The viral nucleic acid was eluted with 50 µL of elution buffer and centrifuged at 8,000 x *g* for 1 minute. The concentration of viral nucleic acid was measured using NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, USA). The purified viral nucleic acid was stored at -70°C until used.

To confirm *Mr*NV infected prawn, the purified viral nucleic acid was used as template for reverse-transcription PCR using the SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* DNA Polymerase (Invitrogen, USA) following the manufacturer's protocol with specific primers (Senapin et al., 2012) as shown in Table 24. The reaction mixture was prepared as shown in Table 25.

Table 24 Primers used in this study

| Primer | Sequence |
|---|---|
| Mr-RdRP-F | 5'- GCA TTT GTG AAG AAT GAA CCG -3' |
| Mr-RdRP-R | 5'- CAT GTT CAAC TTT CTC CAC GT -3' |

Table 25 PCR reaction mixture

| Component | Volume (µL) | Final concentration |
|---|---|---|
| 2X Reaction Mix | 25 | 1X |
| 10 µM forward primer | 1.0 | 0.2 µM |
| 10 µM reverse primer | 1.0 | 0.2 µM |

Table 25 (Continued)

| Component | Volume (µL) | Final concentration |
|---|---|---|
| Template RNA (viral nucleic acid) | 2.0 | |
| SuperScript™ III RT/Platinum™ *Taq* Mix | 2.0 | |
| $dH_2O$ | 19.0 | |
| Total volume | 50.0 | |

The PCR reaction tube was placed into the thermal cycler (Bio-Rad, USA) under condition as described below.

PCR condition :

| | | | |
|---|---|---|---|
| Step 1 | cDNA synthesis | 50°C | for 30 minutes |
| Step 2 | Pre-denaturation | 94°C | for 5 minutes |
| Step 3 | Denaturation | 94°C | for 1 minute |
| Step 4 | Annealing | 55°C | for 45 seconds |
| Step 5 | Extension | 72°C | for 1 minute |
| | Repeat steps 3-5 for 34 cycles | | |
| Step 6 | Final extension | 72°C | for 10 minutes |

The PCR products were subjected to analysis using 1% agarose gel electrophoresis and observed under the UV light using Gel Doc™ XR+ (Bio-Rad, USA). The expected band was then purified using NucleoSpin® Gel and PCR Clean-up Kit (MACHEREY-NAGEL, Germany) following the manufacturer's protocol as described in section 1.3.

### 5.2 Preparation of *Mr*NV

For the immune challenge experiment, *Mr*NV inoculum was prepared following the previously described method (Srisuk et al., 2022). In brief, the *Mr*NV-infected prawn was homogenized using a sterile homogenizer in TN buffer (20-mM Tris-HCl and 0.4-M NaCl, pH 7.4) at a 10% ratio (w/v). Subsequently, the mixture was centrifuged at 11,000 x *g* for 20 minutes at 4°C. After that, the resulting supernatant was then filtered through a 0.45 µm membrane to obtain the *Mr*NV inoculum.

### 5.3 *Mr*NV challenged experiment

The healthy *M. rosenbergii* (1-2 g body weight) were divided into 2 groups, each group consisted of 30 individual prawns. The first group was injected intramuscularly into the third abdominal segment with 50 µL of *Mr*NV ($8.05 \times 10^{11}$ copies/µL nucleic acids) as the challenged group, while the control group was injected with 50 µL of sterile TN buffer. Target organs from each group were collected at 0, 1, 2, 3, 4, 5, 6, and 7 days post injection. Total RNA from collected target organs was extracted using NucleoSpin® RNA Plus (MACHEREY-NAGEL, Germany) following the manufacturer's protocol, as described in section 1.1. The expression of *MrTNF* in target organs after challenged with *Mr*NV was performed using qRT-PCR following the procedure outlined in section 3.3.

The qRT-PCR analysis was conducted in triplicate, and the results were presented as mean ± SD. To determine differences among the experiments, the expressions between challenged group and control group were analyze using Student's *t* test and One-way analysis of variance (ANOVA) with Post Hoc Tukey test. Statistically significant differences were identified at $p<0.05$.

# CHAPTER 4
# RESULTS

## 1. Molecular isolation and identification of full-length cDNA of *MrTNF* by Rapid Amplification of cDNA End (RACE)

In this research, the partial cDNA of *MrTNF* was obtained from our previous study on the transcriptomic of *M. rosenbergii* (Pasookhush et al., 2019). Subsequently, primers specific to the *MrTNF* gene were designed based on the obtained cDNA fragment. These primers were used to conduct the Rapid Amplification of cDNA Ends (RACE) technique, aiming to obtain the complete nucleotide sequence of the *MrTNF* gene.

### 1.1 5'- and 3'-RACE PCR

For 5'-RACE PCR, the specific primer TNF.GSP001 and universal primer mix (UPM) were used to perform the primary PCR. The diluted PCR production of 5'-primary PCR was used as template for 5'-nested PCR using the specific primer TNF.NGSP001 and universal primer mix short (UPM short). The 5'-primary PCR product was about 1000 bp and 5'-nested PCR product was about 750 bp (Figure 16A). For 3'-primary PCR and 3'-nested PCR, the PCR were performed using the specific primers (TNF.GSP002 and TNF.NGSP002, respectively) with the same condition as 5'-RACE PCR. However, the production of 3'-primary PCR was not detected whereas the 3'-nested PCR product was about 750 bp (Figure 16B). The expected 5'- and 3'-PCR products were purified and cloned into pCR®-Blunt II-TOPO® vector and transformed into *E. coli* TOP 10 competent cells for sequencing.

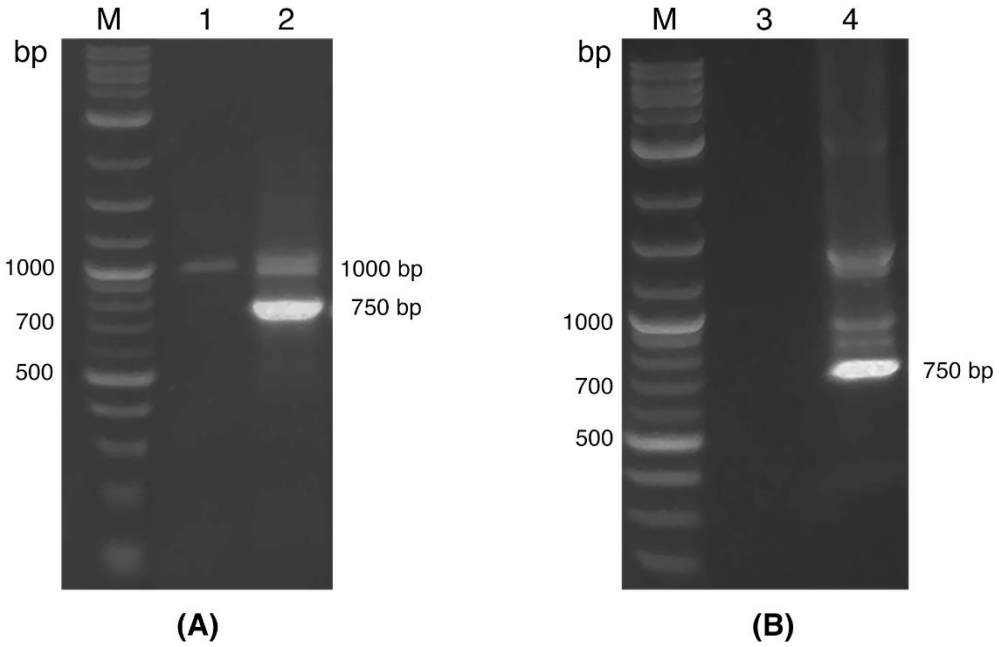



Figure 16 PCR product of *MrTNF* 5'-RACE PCR (A) and 3'-RACE (B).
M = 2-log DNA ladder, Lane 1 = 5'-primary PCR product (1000 bp), Lane 2 = 5'-nested PCR (750 bp), Lane 3 = 3'-primary PCR and Lane 4 = 3'-nested PCR (750 bp).

**1.2 Verification and confirmation of full-length of MrTNF cDNA**

To obtain the full-length cDNA of *MrTNF*, the 5' and 3' ends fragments of *MrTNF* were analyzed by sequencing. These fragments were then combined with a previously obtained partial fragment to generate the full-length cDNA of *MrTNF*. To validate the full-length cDNA of *MrTNF*, the PCR reaction was performed using specific primers (TNF.CDS_F and TNF.CDS_R) designed from *MrTNF* sequence that covered all the coding sequence (CDS). The resulting PCR product was about 1500 bp (Figure 17). The purified PCR product of *MrTNF* coding sequence was cloned into pCR Blunt II-TOPO vector and then transformed into *E. coli* TOP10 competent cells for sequencing.

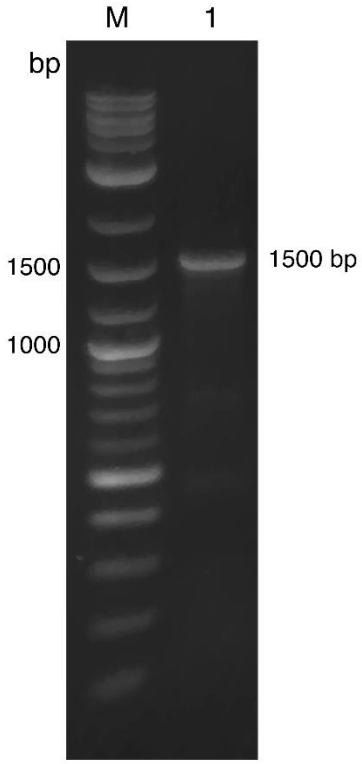



Figure 17 PCR product of the full-length cDNA of *MrTNF*.

M = 2-log DNA ladder, Lane 1 = *MrTNF* PCR product (1500 bp).

**2. Sequence analysis of the full-length cDNA of *MrTNF* by bioinformatic tools**

Upon sequencing and analyzing the 5' and 3' end cDNA fragments of *MrTNF*, the results revealed that the full-length cDNA of *MrTNF* was 1,830 bp, which consisted of 5' untranslated region (5'-UTR) of 396 bp and 3'-UTR of 54 bp. The open reading frame (ORF) was 1,380 bp encoding 459 amino acid residues. However, the poly-adenylation signal at the 3' end was not present (Figure 18). The full-length cDNA sequence of *MrTNF* was deposited into the GenBank database and assigned accession number MW590714.

**2.1 Characterization of *Mr*TNF protein**

The protein *Mr*TNF had a molecular mass of 51.3 kDa and theoretical pI of 9.21. Analysis of domain topology using the SMART tool determined that *Mr*TNF consisted of a transmembrane domain at positions 21 to 43 and a TNF homology domain (THD) at positions 324 to 446. Structure modeling of *Mr*TNF showed that the transmembrane domain was located at the N-terminus, whereas the THD was located at the C-terminus (Figure 19).

Analysis of the THD of *Mr*TNF using BLASTp program demonstrated that *Mr*TNF shared a high identity with *Mn*TNF from *Macrobrachium nipponense* (96.75%), *Mj*TNF from *Marsupenaeus japonicus* (75.61%), *Lv*TNF from *Litopenaeus vanamei* (73.55%), and *Pc*TNF from *Procambarus clarkii* (66.94%). This result suggested that the THD of *Mr*TNF was similar to THDs found in other shrimp species. The multiple sequence alignment of THD was analyzed using MUSCLE tool to compare the sequences of *Mr*TNF with other related species. The results exhibited two conserved cysteine residues in *Mr*TNF among other crustaceans, *Drosophila,* and mammalian TNF (Figure 20).

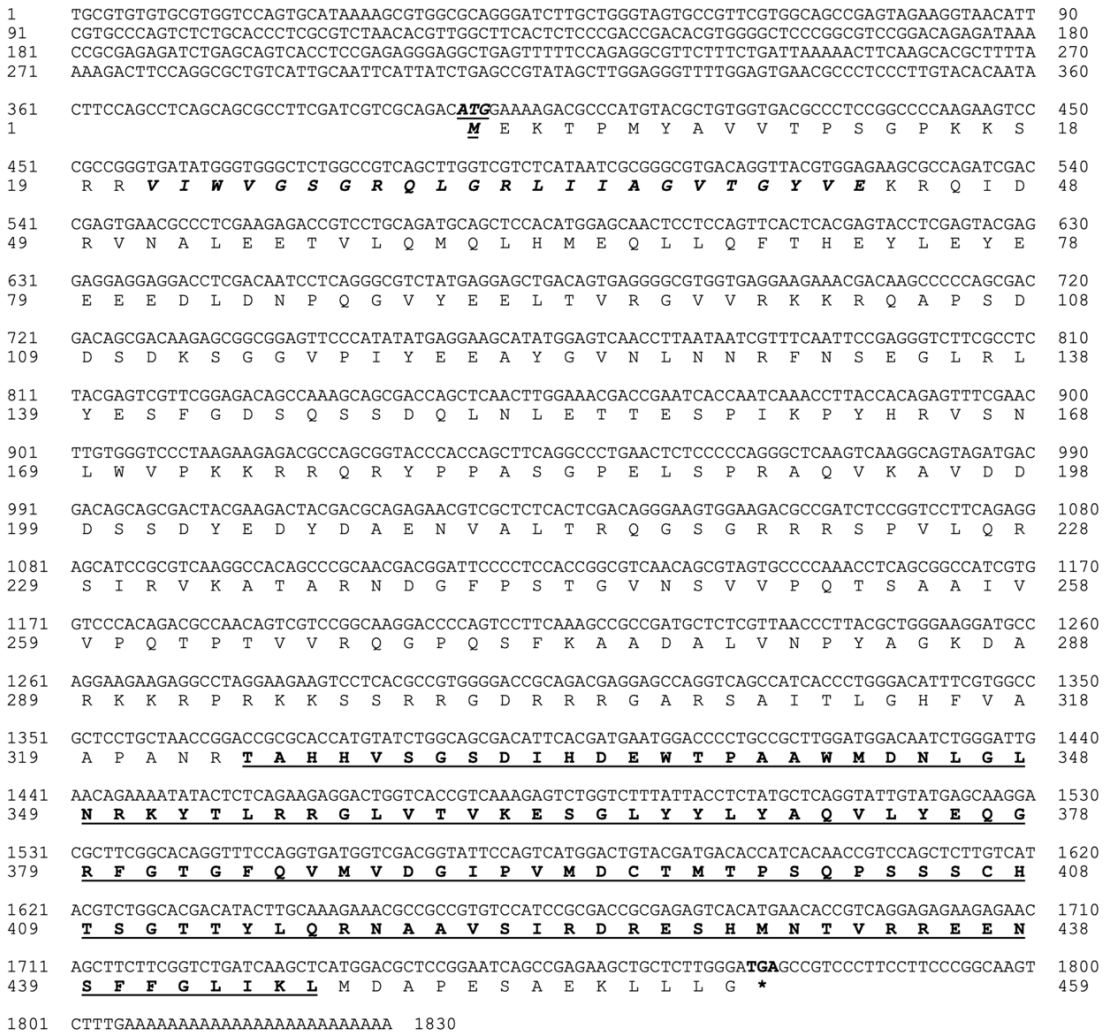


Figure 18 The full-length cDNA of *MrTNF* and the deduced amino acid.

The amino acid sequences in bold and italics indicated the transmembrane domain structure. The amino acid sequences in bold and underlined indicated the TNF homology domain (THD) structure.

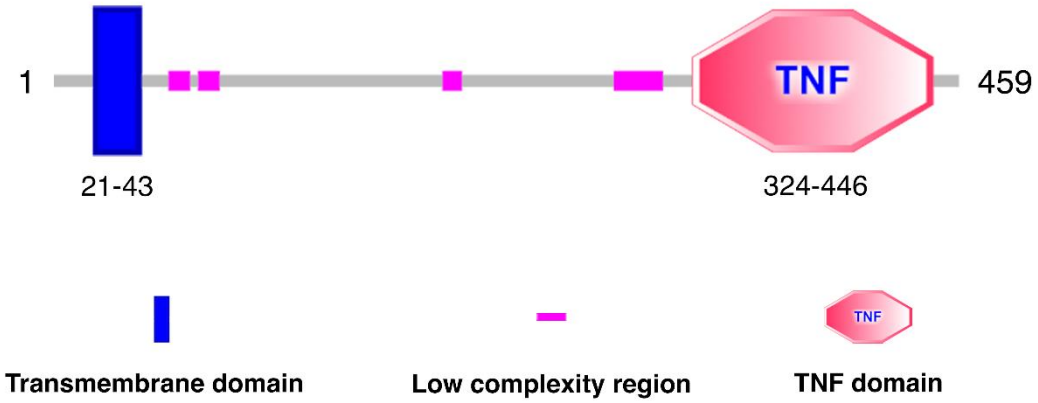



Figure 19 Schematic representation of the structural analysis of *Mr*TNF. *Mr*TNF contained a transmembrane domain at N-terminus and TNF homology domain (THD) at C-terminus.

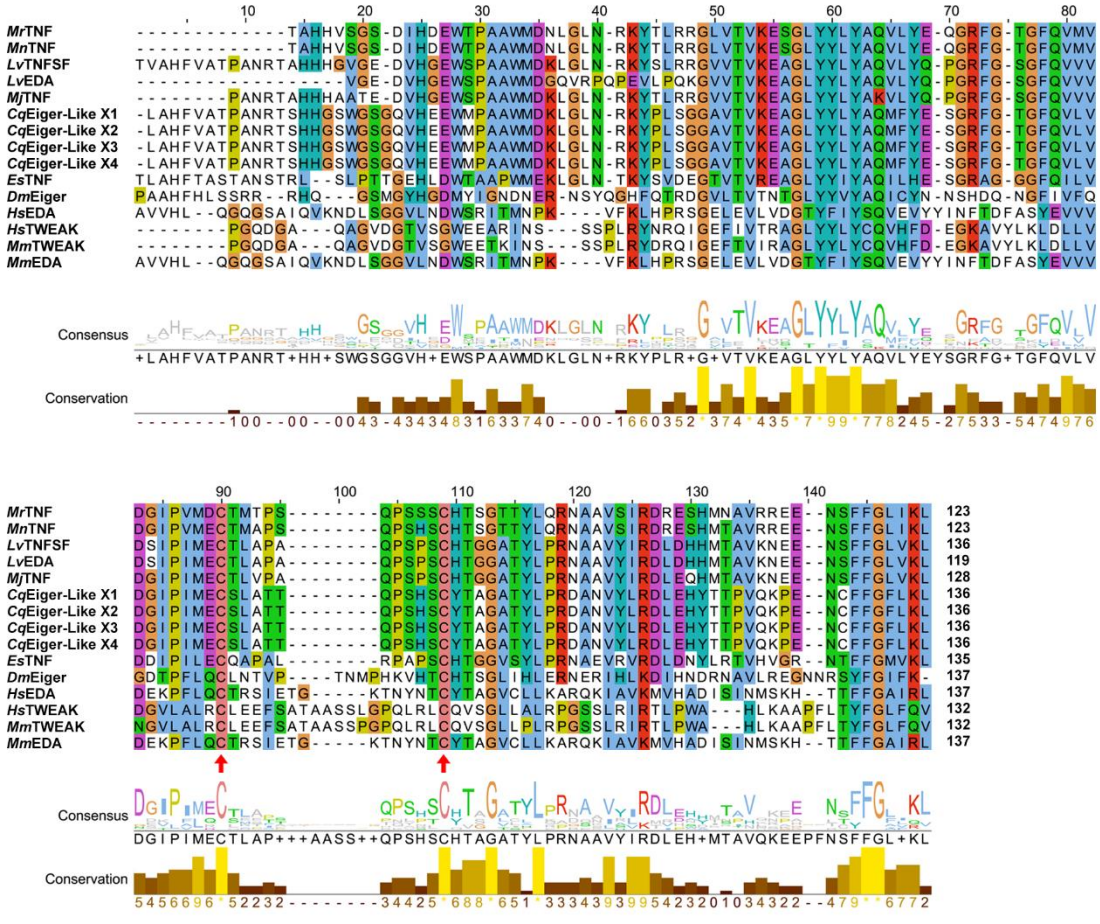



Figure 20 The multiple sequence alignment of amino acid sequences of *Mr*TNF C-terminus THD and other TNFs THD.

The conserved cysteines (C) were shaded in pink with red arrow. The identical or highly conserved residues were colored in a similar shade. Accession numbers; *M. rosenbergii* (*Mr*TNF, MW590714), *M. nipponense* (*Mn*TNF, QCS40507), *M. japonicus* (*Mj*TNF, BAJ10320), *L. vanameii* (*Lv*EDA, XP_027209569; *Lv*TNFSF, AEK86525), *Cherax quadricarinatus* (*Cq*Eiger-Like X1, XP_053644226*; Cq*Eiger-Like X2, XP_053644227*; Cq*Eiger-Like X3, XP_053644229*; Cq*Eiger-Like X4, XP_053644230), *E. sinensis* (*Es*TNF, UYL04284), *D. melanogaster* (*Dm*Eiger, NP_724878), *Homo sapiens (Hs*EDA, Q92838; *Hs*TWEAK, O43508*),* and *Mus musculus (Mm*EDA, O54693; *Mm*TWEAK, O54907).

Furthermore, pairwise alignment of TNF proteins among crustaceans, invertebrates, and vertebrates were aligned to generate an identity and similarity matrix. The analysis demonstrated that *Mr*TNF exhibited a high level of conservation with crustacean TNFs, representing a similarity matrix score of 93.39% with *Mn*TNF and 54.28% with *Es*TNFSF, for the highest and lowest similarity, respectively (Figure 21)

| | *Mr*TNF | *Mn*TNF | *Mj*TNF | *Lv*TNF | *Lv*EDA | *Es*TNF | *Dm*Eiger | *Hs*TWEAK | *Hs*EDA | *Mm*TWEAK | *Mm*EDA |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Mr*TNF | | 93.39 | 60.82 | 58.73 | 53.88 | 54.28 | 31.79 | 22.18 | 25.45 | 22.18 | 25.66 |
| *Mn*TNF | 90.19 | | 59.92 | 58.09 | 53.71 | 52.98 | 32.60 | 22.06 | 24.95 | 22.27 | 25.15 |
| *Mj*TNF | 49.59 | 48.50 | | 83.27 | 75.93 | 49.53 | 30.75 | 22.27 | 25.90 | 22.48 | 25.90 |
| *Lv*TNFSF | 47.02 | 46.39 | 80.04 | | 91.38 | 54.76 | 30.69 | 21.72 | 26.21 | 21.93 | 26.21 |
| *Lv*EDA | 42.05 | 41.52 | 72.02 | 90.35 | | 50.75 | 27.95 | 20.32 | 24.34 | 20.32 | 24.34 |
| *Es*TNFSF | 38.72 | 37.81 | 36.07 | 42.33 | 38.30 | | 30.62 | 21.90 | 26.22 | 21.90 | 26.59 |
| *Dm*Eiger | 15.49 | 15.90 | 15.08 | 15.44 | 14.26 | 14.37 | | 23.79 | 28.77 | 23.09 | 28.31 |
| *Hs*TWEAK | 11.30 | 11.13 | 11.55 | 11.68 | 11.55 | 10.85 | 11.55 | | 22.52 | 93.57 | 22.28 |
| *Hs*EDA | 11.11 | 10.58 | 11.75 | 11.84 | 11.32 | 10.86 | 13.47 | 10.89 | | 21.53 | 94.63 |
| *Mm*TWEAK | 12.13 | 12.16 | 12.39 | 12.50 | 12.15 | 11.63 | 11.09 | 89.16 | 10.64 | | 21.29 |
| *Mm*EDA | 11.52 | 10.98 | 11.95 | 12.04 | 11.51 | 11.42 | 13.24 | 10.89 | 94.63 | 10.40 | |

% Identity of TNFs protein

% Similarity of TNFs protein

Figure 21 The pairwise alignment of *Mr*TNF and other TNFs.

The pairwise similarity were shown in the upper right corner and the pairwise identity were shown in the lower left corner. Accession numbers; *M. rosenbergii* (*Mr*TNF, MW590714), *M. nipponense* (*Mn*TNF, QCS40507.1), *M. japonicus* (*Mj*TNF, BAJ10320.1), *L. vanameii* (*Lv*EDA, XP_027209569.1; *Lv*TNFSF, AEK86525.1), *E. sinensis* (*Es*TNF, UYL04284.1), *D. melanogaster* (*Dm*Eiger, NP_724878), *H. sapiens (Hs*EDA, Q92838; *Hs*TWEAK, O43508*),* and *M. musculus (Mm*EDA, O54693; *Mm*TWEAK, O54907).

2.2 Phylogenetic tree

To construct a phylogenetic tree, the conserved TNF homology domain (THD) of the TNF superfamily (TNFSF) from various species, such as crustaceans, invertebrates, vertebrates, and mammals, were aligned with MUSCLE tool and constructed a phylogenetic tree using maximum likelihood (ML) method with Jones-Taylor-Thornton (JTT) matrix-based model with replicate of bootstrap of 1000. The result indicated that the TNFSF members were distinguished in 16 subgroups. *Mr*TNF was closely related to *Mn*TNF from *M. nipponense* and clustered together with other TNFSF of crustaceans, invertebrates, and then merged into TNFSF12 subgroub. (Figure 22).

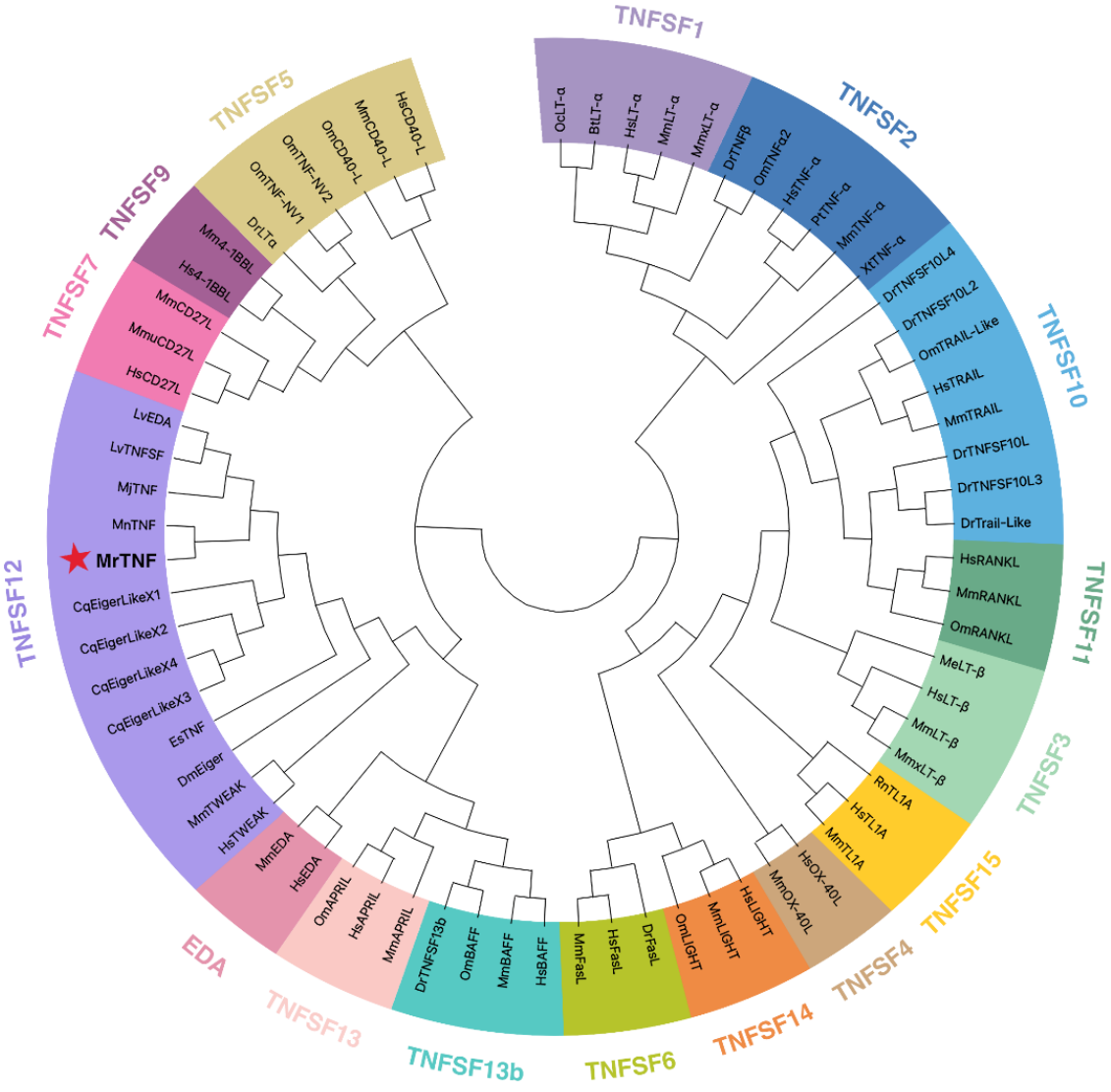



Figure 22 The phylogenetic tree analysis of TNF homology domain (THD) from various TNFSF member using ML-method with bootstrap of 1000.

Accession numbers; *M. rosenbergii* (*Mr*TNF, MW590714), *M. nipponense* (*Mn*TNF, QCS40507), *M. japonicus* (*Mj*TNF, BAJ10320), *L. vanameii* (*Lv*EDA, XP_027209569; *Lv*TNFSF, AEK86525), *C. quadricarinatus* (*Cq*Eiger-Like X1, XP_053644226; *Cq*Eiger-Like X2, XP_053644227; *Cq*Eiger-Like X3, XP_053644229; *Cq*Eiger-Like X4, XP_053644230), *E. sinensis* (*Es*TNF, UYL04284), *D. melanogaster* (*Dm*Eiger,

NP_724878), *Bos Taurus* (*Bt*LT-α, NP_001013419), *Danio rerio* (*Dr*TNFβ, NP_001019618; *Dr*TNFSF13b, NP_001107062; *Dr*FasL, NP_001036166; *Dr*Trail-Like, AAG47640; *Dr*TNFSF10L, NP_571918; *Dr*TNFSF10L2, NP_001002593; *Dr*TNFSF10L3, NP_001036178; *Dr*TNFSF10L4, NP_001013301;), *H. sapiens* (*Hs*APRIL, O75888; *Hs*BAFF, Q9Y275; *Hs*4-1BBL, P41273; *Hs*CD40-L, P29965; *Hs*CD27L, P32970; *Hs*FasL, P48023; *Hs*EDA, Q92838; *Hs*TNF-α, NP_000585; *Hs*TRAIL, P50591; *Hs*TWEAK, O43508; *Hs*RANKL, O14788; *Hs*TL1A NP_005109; *Hs*LT-α, NP_000586; *Hs*LT-β, NP_002332; *Hs*LIGHT, O43557; *Hs*OX-40L, P23510), *Macaca mulatta* (*Mmu*CD27L, XP_001088935), *Rattus norvegicus* (*Rn*TL1A, NP_665708), *Marmota monax* (*Mm*xLT-α, AAF34868; *Mm*xLT-β, AAF34865), *M. musculus* (*Mm*APRIL, Q9D777; *Mm*BAFF, Q9WU72; *Mm*4-1BBL, P41274; *Mm*CD27L, O55237; *Mm*CD40-L, P27548; *Mm*EDA, O54693; *Mm*FasL, P41047; *Mm*TRAIL, P50592; *Mm*RANKL, O35235; *Mm*TL1A, NP_796345; *Mm*TWEAK, O54907; *Mm*TNF-α, NP_038721; *Mm*LT- β, NP_032544; *Mm*LT-α, NP_034865; *Mm*LIGHT, Q9QYH9*Mm*OX-40L, P43488), *Macropus eugenii* (*Me*LT-β, AAD41774), *Oncorhynchus mykiss* (*Om*APRIL, O75888; *Om*BAFF, NP_001118036; *Om*CD40-L, NP_001118138; *Om*LIGHT, NP_001118039; *Om*TNF-NV1, NP_001118041; *Om*TNF-NV2, ABC84588; *Om*TNFα2, NP_001117846; *Om*TRAIL-Like, NP_001118037), *Oryctolagus cuniculus* (*Oc*LT-α, P10154), *Pan troglodytes* (*Pt*TNF-α, NP_001038976), and *Xenopus tropicalis* (*Xt*TNF-α, NP_001107143).

### 3. Tissue distribution of *MrTNF* in healthy prawns

To investigate the expression of *MrTNF* in healthy adult *M. rosenbergii*, the total RNA from various tissues were examined using qRT-PCR with specific primers (TNF.qPCR_F and TNF.qPCR_R) and primer specific to an elongation factor 1 alpha gene (EF1αF and EF1αR) as an internal control gene. The relative expression of *MrTNF* was normalized with EF1α. The result revealed that *MrTNF* mRNA was predominantly expressed in the intestine approximately 0.0045-fold, followed by muscle, stomach, and heart, with approximately 0.0029, 0.0025, and 0.0024-fold, respectively. In contrast, the expression of *MrTNF* mRNA was lower in the gills, hepatopancreas, and hemocyte with approximately of 0.0009, 0.0003, and 0.00002-fold, respectively (Figure 23).

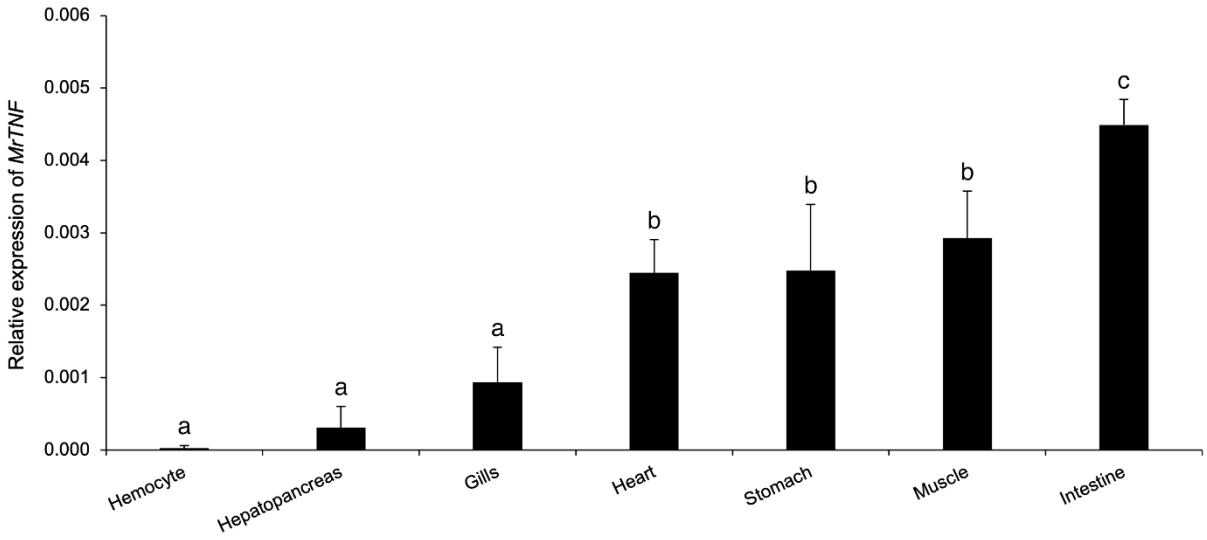



Figure 23 Tissue distribution of *MrTNF* gene in healthy prawns *M. rosenbergii*. Results were shown as the mean ± SD (n=3). Each bar represented mean relative expression, whereas the error bars represented standard deviation. Bars with different alphabet denoted significant difference at $p < 0.05$.

**4. The expression level of *MrTNF* in immune challenged with *A. hydrophila***

To explore the function of *MrTNF* in the innate immune system, the expression level of *MrTNF* was analyzed in various organs, including hemocyte, muscle, intestine, and stomach, after immune challenge with *A. hydrophila*. The findings indicated that the expression level of *MrTNF* in hemocyte was significantly increased at 6 and 24 hpi (Figure 24A). Moreover, *MrTNF* was up-regulated in muscle at 12 hpi, but significantly decreased at 24 hpi, and returned to the basal level from 36 hpi onwards (Figure 24B). In the intestine, the expression level of *MrTNF* was considerably elevated from 3 to 24 hpi, and then slightly decreased to the basal level after 36 to 48 hpi (Figure 24C). Additionally, the expression level of *MrTNF* in the stomach was significantly up-regulated at 24 and 36 hpi (Figure. 24D). To determine the presence and growth of A. *hydrophila*, bacterial colony counts were conducted in the hemolymph of prawns at all tested timepoints. The results revealed that the bacterial counts at 3, 6, 12, 24, 36, and 48 hpi were $8.2x10^4$, $7.8x10^4$, $6x10^4$, $1.2x10^5$, $1.7x10^5$, and $9.3x10^4$ CFU/mL, respectively.

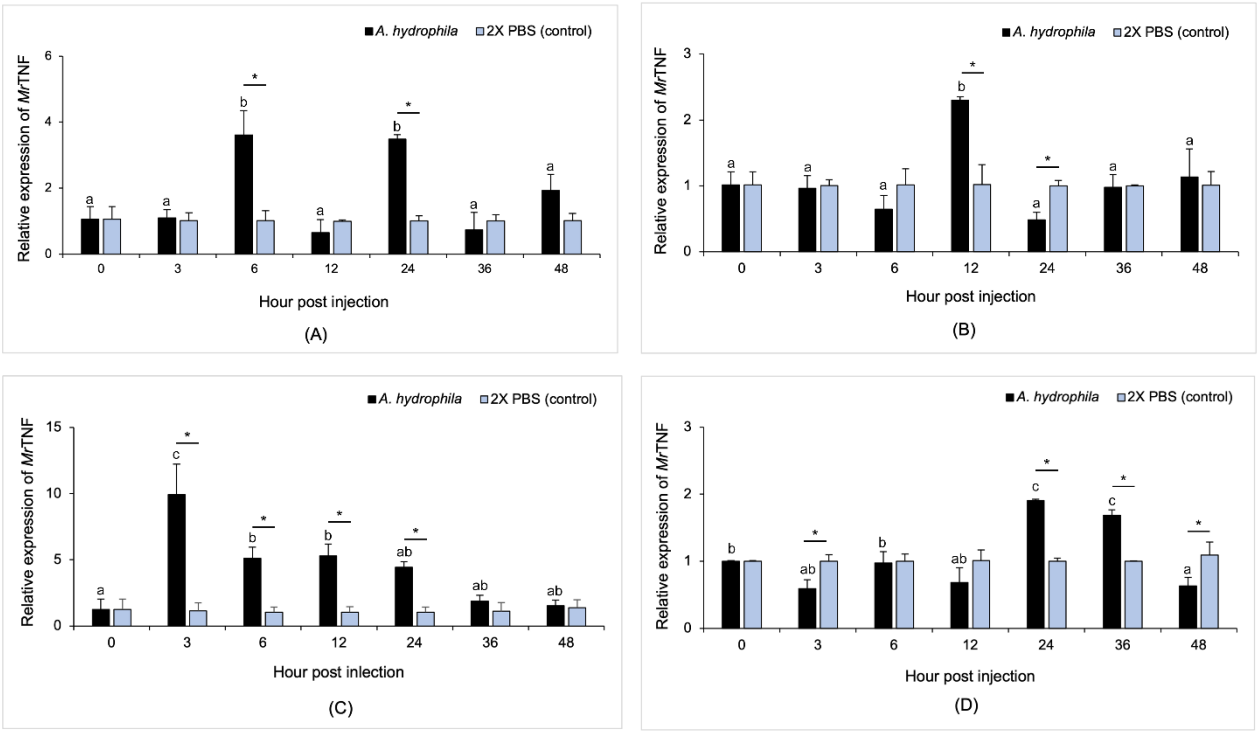



Figure 24 The expression level of *MrTNF* mRNA in several organ after immune challenge experiment with *A. hydrophila.*

The expression level of *MrTNF* in hemocyte (A), muscle (B). intestine (C) and stomach (D) after immune challenge experiment with *A. hydrophila*. Results were shown as the mean ± SD (n=3). Each bar represented mean relative expression, whereas the error bars represented standard deviation. Bars with different alphabets denoted the different expression level between *A. hydrophila* injection group at each time-point. Asterisks indicated significant differences at $p$ <0.05 between *A. hydrophila* group and 2X PBS (control) group.

**5. The expression level of *MrTNF* in immune challenged with *Mr*NV**

To study the regulation of *MrTNF* expression during viral infection, prawns were subjected to challenge with *Mr*NV, and the expression of *MrTNF* was examined in serval tissue, specifically in the muscle, gills, and hepatopancreas. The results indicated that *MrTNF* expression level in muscle was significantly up-regulated at 2 days post injection but significantly down-regulated at 3 to 7 days post injection (Figure 25A). In gills, the expression of *MrTNF* was slightly up-regulated at 4 days post injection and significantly up-regulated at 6 to 7 days post injection (Figure 25B). In hepatopancreas, the expression level of *MrTNF* was significantly up-regulated at 3, 5, and 7 days post injection (Figure 25C).

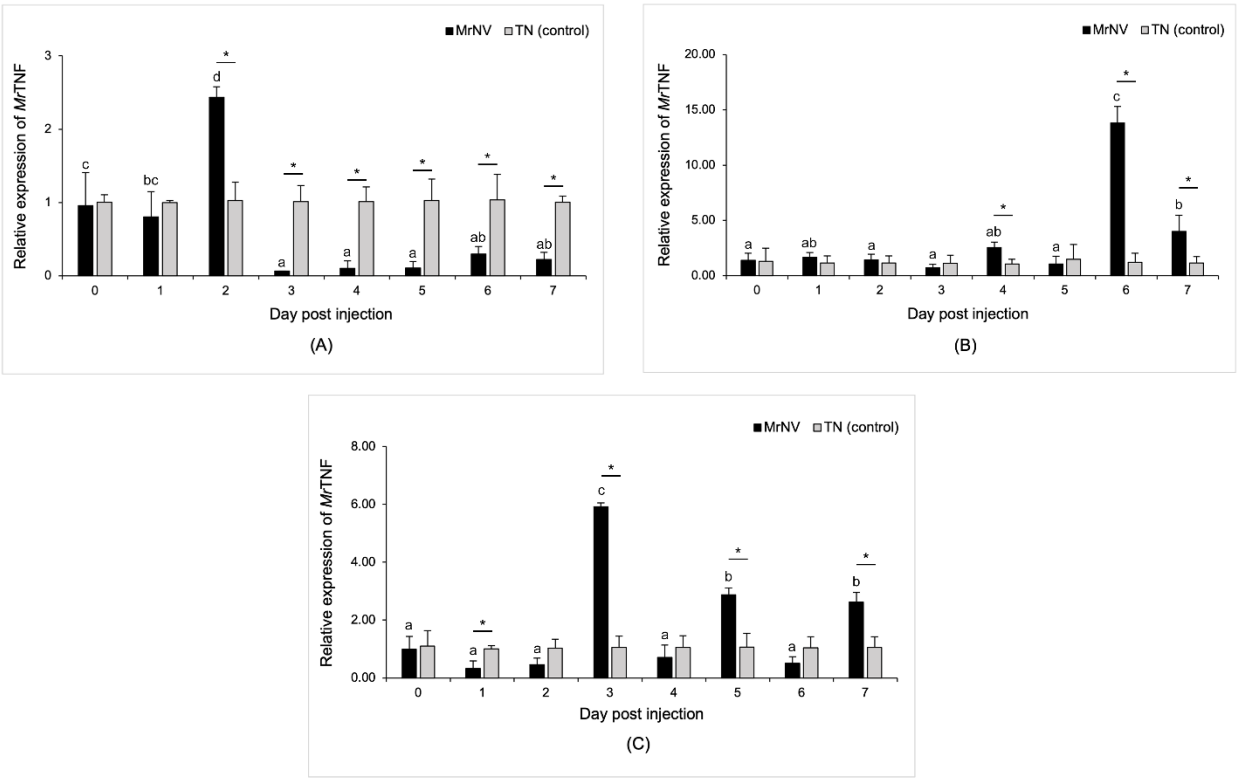



Figure 25 The expression level of *MrTNF* in muscle (A), gills (B), and hepatopancreas (C) after immune challenge with *Mr*NV.

Results were shown as the mean ± SD (n=3). Each bar represented mean relative expression, whereas the error bars represented standard deviation. Bars with different alphabets denoted significant differences of the expression level of *Mr*NV-injected group at each time-point. Asterisks indicated significant differences at $p < 0.05$ between *A. hydrophila* group and TN (control) group.

# CHAPTER 5
# DISCUSSION AND CONCLUSION

Tumor necrosis factor (TNF) is a cytokine belonging to the tumor necrosis factor superfamily (TNFSF) that plays a crucial role in the immune system of vertebrate, including cell inflammation, cell proliferation, and cell death (Goetz, Planas, & MacKenzie, 2004). Within the TNFSF, there are 19 known ligands extensively studied for their involvement in various physiological processes, differing in binding specificity and affinity for their receptors (Bodmer, Schneider, & Tschopp, 2002). TNF was first discovered in 1970s as a factor causing tumor cell death in mice (Old, 1988). Since then, TNF has been found in various vertebrate species, including human, mammals, and teleost (Hirono, Nam, Kurobe, & Aoki, 2000; Locksley et al., 2001). Invertebrates, a diverse group of animals lacking a backbone, have immune systems distinct from those of vertebrates. Invertebrate immune responses rely on innate immunity, which is a non-specific response to pathogens. Recently, TNFSF members have been identified in invertebrates such as arthropods, echinoderms, mollusks, and crustaceans (Hibino et al., 2006; Huang et al., 2022; Sun et al., 2014). The TNFSF in invertebrates highlights the role in immune response by activating the JNK, Toll, and IMD pathway (Tang, Li, Wang, Wu, & Liu, 2019)

Recently, TNFSF has been reported in four species of shrimp, such as *M. japonicus*, namely *Mj*TNF (Mekata et al., 2010), *L. vannamei* which were *Lv*TNFSF, *Lv*TNSFR, and *Lv*LITAF (Wang et al., 2012), *P. clarkii*, namely *Pc*TNF (Calderón-Rosete et al., 2018), and *M. nipponense*, namely *Mn*TNF (Qin et al., 2019). In this study, the complete cDNA sequence of the tumor necrosis factor (TNF) gene in the giant freshwater prawn *M. rosenbergii* was successfully identified for the first time and named as *MrTNF*. The full-length cDNA of *MrTNF* was 1,830 bp, with an open reading frame (ORF) of 1,380 bp encoding 459 amino acids. *Mr*TNF protein consisted of a transmembrane domain and a TNF homology domain (THD). The *Mr*TNF sequence analysis was deposited in the GenBank database under accession No. MW590714. The

structure of *Mr*TNF was highly similar to *Mn*TNF, which lacked a signal peptide (Qin et al., 2019). According to multiple sequence alignment results of the THD of *Mr*TNF and other THDs, it was found that the structure of THD in *Mr*TNF contained two conserved cysteine residues that were similar to other crustaceans, invertebrates, and mammals. The presence of two conserved cysteine residues in THD of *Mr*TNF was essential for its signaling activity via the TNF receptor (TNFR), which contains cysteine-rich domains (CRD) for binding to TNF (MacEwan, 2002; Ware, 2003). The pairwise alignment of *Mr*TNF protein revealed that *Mr*TNF shared similarity to *Mn*TNF, *Mj*TNF, *Lv*TNFSF, *Lv*EDA, *Es*TNFSF, and *Dm*Eiger with 93.39%, 60.82%, 58.88%, and 31.79%, respectively. Furthermore, *Mj*TNF was reported to share 35.5% similarity with *Dm*Eiger (Mekata et al., 2010).

To study the relationship between *Mr*TNF and TNFs in other living organisms, the conserved TNF domain of *Mr*TNF and other species were analyzed through the multiple sequence alignment to construct the phylogenetic tree. The result of the phylogenetic tree analysis indicated that *Mr*TNF was closely related to *Mn*TNF from *M. nipponense*. However, *Mr*TNF was clustered in different subclade with the TNF of penaeid prawns, *M. japonicus*, and *L. vannamei*. Moreover, *Mr*TNF and TNF of invertebrates were classified into the TNFSF12 subgroup, along with TNF of mammals, *Hs*TWEAK from humans and *Mm*TWEAK from mice. Similar to previous study, *Mn*TNF and *Mj*TNF were also grouped with vertebrate TNFSF, *Hs*TWEAK and *Mm*TWEAK under TNFSF12 subgroup (Qin et al., 2019). TNF-related weak inducer of apoptosis (TWEAK) also known as TNFSF12 that could induce a variety of biological activities by stimulating fibroblast growth factor-inducible-14 (Fn14) related to activation of angiogenesis, cell proliferation, cellular differentiation, and cell death (Anany et al., 2018). TWEAK might play a functional role in promoting cytotoxicity in monocytes and NK cells (Locksley et al., 2001). Although there are similarities between TNFSF in invertebrates and vertebrates, the phylogenetic tree analysis in this study did not provide definite evidence of their evolutionary origins. Further research is necessary to gain deeper insights and

enhance our understanding of the evolutionary relationships among TNF proteins in various organisms.

The difference between TNF in vertebrates and invertebrates is the type of cells that synthesize TNF in response to infection or other stimuli. TNF in vertebrates is produced by a variety of immune cells such as monocytes, macrophages, dendritic cells, T cells, B cells, and NK cells (Croft, Benedict, & Ware, 2013). In contrast, in invertebrates TNF is primarily produced by hemocytes, the equivalent of white blood cells in vertebrates, which are present in the circulatory system and responsible for other immune functions (Jiravanichpaisal et al., 2006). To investigate the distribution of *MrTNF* in different tissues of healthy *M. rosenbergii*, the expression levels of *MrTNF* were examined using qRT-PCR. In this study, the relative expression of *Mr*TNF was normalized to elongation factor 1 aphla (EF1$\alpha$) serving as an internal control gene. Among the various tissues examined, the highest expression level of *MrTNF* was found in the intestine, which significantly differed from other tissues. The expression levels of *MrTNF* were prominently detected in the muscle, stomach, and heart, indicating higher expression in these tissues. In contrast, lower expression levels were observed in the gills, hepatopancreas, and hemocytes. This expression pattern aligns with the observed expression patterns of MjTNF and LvTNFSF, which exhibited higher expression levels in the muscle and stomach, but lower expression levels in the hepatopancreas and hemocytes (Mekata et al., 2010; Wang et al., 2012). Despite that, *MnTNF* exhibited higher expression levels in the nerve cord and hemocyte, but lower expression level in the hepatopancreas (Qin et al., 2019). These results indicated that TNF gene expression may vary across species.

To examine the role of TNF in the immune system of crustaceans to various types of their response to bacterial, and viral infections. *A. hydrophila* is a pathogenic bacterium that found in shrimp and other crustaceans (Zeng, 2020). The expression of *MrTNF* was observed to be increased in the hemocyte, muscle, intestine, and stomach upon *A. hydrophila* challenge. The results revealed that the expression of *MrTNF* were up-regulated in hemocyte at 6 and 12 hpi, in muscle at 12 hpi, in intestine at 3, 6, and

12 hpi, and in stomach at 24 and 36 hpi after challenged with *A. hydrophila*, which similar to the up-regulation of *EsTNFSF* expression levels in hemocyte at 2, 6, 12, and 24 hpi after *Vibrio* sp. stimulation (Huang et al., 2022). *MnTNF* expression levels were up-regulated in gills at 6 hpi after *Aeromonas veronii* stimulation (Qin et al., 2019). *MjTNF* expression levels were up-regulated in gills at 4 hpi with *V. penaeicida* stimulation and at 2 hpi with LPS stimulation. Furthermore, the expression levels of *MjTNF* were found to be up-regulated in the lymphoid organ at 4 hours post-infection (hpi) following PGN stimulation, as well as at 4 and 12 hpi following poly I:C stimulation. However, no significant difference in *MjTNF* expression levels was observed in cells stimulated with LPS (Mekata et al., 2010). *LvTNFSF* expression levels were found to be increased in the gills, intestine, and hepatopancreas after *S.aureus* stimulation, but down-regulated in the intestine at 9 hpi with *V. alginolyticus* stimulation. *LvTNFSF* expression levels did not change in the intestine after challenge with *C. albicans*, but it was found to be up-regulated in gills at 9 and 12 hpi, and in the hepatopancreas at 9 hpi (Wang et al., 2012). In addition, viruses are also pathogens that can stimulate immune responses in crustaceans. *Macrobrachium rosenbergii* nodavirus (*Mr*NV) is an RNA virus that infects the freshwater prawn *M. rosenbergii* and can lead to high mortality rates in infected prawns, particularly in the larval and post-larval stages (Ho et al., 2018). In this study, the expression of *MrTNF* was investigated in response to *Mr*NV challenge to understand its involvement in immune response. The findings indicated that the expression level of *MrTNF* was up-regulated in the muscle on days 2 but was significantly down-regulated on days 3 to 7 after *Mr*NV challenge. In the gills, the expression level of *MrTNF* was up-regulated on days 4, 6, and 7 after *Mr*NV challenge. In the hepatopancreas, the expression level of *MrTNF* was up-regulated on days 3, 5, and 7 after *Mr*NV challenge. Similarly, *LvTNFSF* expression level in the intestine was down-regulated at 3, 9, 12, and 24 hpi after WSSV challenge, while its expression in the gills and hepatopancreas was up-regulated (Wang et al., 2012). These results suggest that *MrTNF* may play an important role in the immune response of *M. rosenbergii* against both Gram-negative bacteria and virus.

In summary, this study presented the cloning and characterization of the tumor necrosis factor gene in freshwater prawn *M. rosenbergii* (*MrTNF*) for the first time. The *Mr*TNF structure was found to be similar to other reported TNFs, which contained a TNF homology domain (THD) that closely related to other crustaceans. The expression level of *MrTNF* was found to be higher in the intestine muscle and stomach of healthy prawn. Upon *A. hydrophila* challenge, *MrTNF* was up-regulated in the hemocyte, muscle, intestine, and stomach. In the case of *Mr*NV challenge, *MrTNF* was up-regulated in muscle, gills, and hepatopancreas. The study provided new insights into the expression pattern of *MrTNF* in various organs and highlighted the importance of further research into crustacean immune systems to understand the mechanisms underlying their immune responses against bacterial and viral infections. This research could facilitate in the development of more effective treatments for crustacean diseases in the future.

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