Characterisation of PAMP-PRR interaction and the immune response in Nile tilapia

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Declaration

I hereby declare that, this thesis has been composed entirely by me, except where specifically acknowledged. The work described in this thesis has been done independently by me and has not been submitted for any other degree.

Savitree Ritchuay

Signature: _____

Signature of supervisor: _____

Date: _____

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Savitree Ritchuay

List of abbreviations

°C	Degree Celsius
%	Percentage
µg	Microgram
µl	Microliter
µm	Micrometers
µM	Micromolar
µm	Micrometre/micron
AChE	Acetylcholinesterase
Bp	Base pair
CD14	Cluster of differentiation 14
cDNA	Complementary DNA
CKS	Chicken serum
Cm	Centimetre
CO_2	Carbon dioxide
Ct	Cycle threshold
DMEM	Dulbecco's Modified Eagle's Medium
dsRNA	Double-stranded RNA
E	Efficiency of qPCR
e.g.	Exempli gratia (example)
ER	Endoplasmic reticulum
et al	And others
FAO	Food and Agriculture Organization
FBS	Fetal bovine serum
FCS	Forward scatter
FITC	Fluorescein Isothiocyanate
g	Gravity
g	Gram
h	hour
IFN	Interferon
IL-10	Interleukin-10

IL-1β	Interleukin-1-beta
IL-6	Interleukin-6
IRF	Interferon transcription factor
L	Litre
LPS	Lipopolysaccharide
m^2	Square meter
mg	Milligrams
min	Minute
mL	Milliliters
mRNA	messenger Ribonucleic acid
N	Number
NCBI	National Center for Biotechnology Information
NFκ-β	Nuclear factor kappa-b
Nm	Nanometre
PAMP	Pathogen associated molecular pattern phosphate
PBS	Phosphate Buffered Saline
PGE_2	Prostaglandin E_2
PGN	Peptidoglycan
Poly(I:C)	Polyinosinic and polycytidylic acid; Poly(I:C)
PLL	Poly-l-lysine
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
r^2	Coefficient of determination
RLR	RIG-like receptor
RNA	Ribonucleic acid
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
SSC	Side scatter
TAE	Tris acetate EDTA
TGF-β	Transforming growth factor-beta
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha

Trypsin /EDTA	Trypsin in 0.01 % ethylene diamine tetra acetic acid
UV	Ultraviolet
V	Volt
v/v	Volume/Volume
VIPERIN	Virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible
w/v	Weight/Volume

Abstract

The innate immune system is the first line of host defense against invading pathogens across the entire animal kingdom. Pathogen recognition and effective response are essential to survive in a microbe-rich environment that often characterizes certain types of tilapia aquaculture. In this thesis, we developed a platform to study the innate immune response of the Nile tilapia (*Oreochromis niloticus*). Specifically, a macrophage model system was characterised and used to explore PAMP-PRR interactions. Moreover, the basal expression of targeted innate immunity gene was measured in different tissues of tilpia, cultured under different aquaculture environments.

The macrophage primary cell culture was used to characterize PAMP-PRR interactions after stimulation with upPGN or dsRNA at the level of mRNA transcription of cytokines and antiviral related genes using absolute qPCR and secreted prostaglandins in the cell supernatant. A phylogenetic study of the target genes revealed conservation of Nile tilapia innate immunity genes across different species and all extant teleosts. Head kidney derived macrophages from Nile tilapia were optimally cultured and stimulated with PAMPs over specific time periods. Results revealed moderate levels of secreted PGE_2 in culture media but no change upon PGN stimulation. Cytokine mRNAs were generally upregulated although high levels of mRNAs were found in basal state cells. Granulomas were observed during cell culture suggestive of chronic infection with intracellular parasites. Mycobacterium detection using PCR based method was able to detect mycobacterium DNA in macrophages and tissues samples of Nile tilapia (*Oreochromis niloticus*).

The qPCR tools developed were used to examine the tissue-specific gene expression in tilapia cultured in Thailand. Data suggests that red hybrid tilapia (*O.niloticus* X *O. mossambicus*) are potentially more sensitive to culture conditions, particularly in polyculture husbandry systems in comparison to Nile tilapia (*Oreochromis niloticus*).

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Chapter 1

General Introduction

1.1 Tilapia

1.1.1 Tilapia in aquaculture

Tilapia is the common name given to more than 70 freshwater and some brackish water fish species and strains that are members of the family 'Cichlidae' ;order 'Perciformes' . Tilapias are native fish in Africa and they are now widely distributed worldwide (Asia, South and Central America, Southern India and Israel) . Tilapia aquaculture stated with the culture of Nile tilapia (*Oerochromis niloticus*) over 4,000 years ago in ancient Egypt. While the initial worldwide distribution of tilapias took place during the 1940s and 1950s with the distribution of the Mozambique tilapia (*O. mossambicus*) it was not until the1960s that Nile tilapia (*O. niloticus*) was introduced to Asia from Japan to Thailand and then exported to the Philippines and became the most important commercial species of tilapia (Rocky 2005). Thereafter, Nile tilapia was introduced to the United states in 1974 from Brazil. Finally, in 1978 Nile tilapia was introduced to China, which became the largest tilapia producer of the world (Rakocy 2005). Although there are over 70 species and strains of tilapia, only a few species are suitable and popular for aquaculture production, namely *O. niloticus, O. aureus, O. mossambicus* and *O. hornorum where O. niloticus* has the highest production (Beveridge and McAndrew 2012) (Fig 1.1).

Figure 1.1 Nile tilapia (*Oreochromis niloticus*) (Ritchuay, 2018)

The primary aim when tilapia was introduced to developing countries was to support sustainable food production for local consumption. Since then, tilapia production has gradually expanded worldwide as it is suitable for culture in a wide range of farming environments such as ponds, floating cages, tanks and raceways. Tilapia is known or referred to as the "aquatic chicken" because of its high protein content, rapid growth, adaptability and breeds easily in captivity. Beside, tilapia species tolerate significant environmental and water quality fluctuations. It can tolerate low temperatures of 6-10 °C for short periods, and high temperatures of 35-42 °C. It can grow between 20-35 °C (Zhao et al 2015) and suitable temperature for spawning range from 25-30 °C. Moreover, it can be cultured in freshwater, brackish water and seawater (El-Sayed 2006). For instances, Nile tilapia can be cultured in tropical freshwater and tolerates salinity up to 15 ppt. In tropical freshwater systems, the most common popular cultured tilapia is *O. niloticus*, whereas *O. aureus* is suitable for subtropical freshwater as it can tolerate colder water than *O. niloticus.* Moreover, a hybrid strains of tilapia have been developed in order to improve the performance or to select for particular trains. Examples include strain generated from cross-breeding between *O. mossabicus* and *O. hornorum* to generate all male fry, *O.niloticus* and *O.aureus* to increase low temperature tolerance and a strain generated from cross-breeding *O.niloticus* and *O. mossambicus* that exhibits red colour trait (reviewed in Beveridge and McAndrew 2012).

Due to the rapid growth of aquaculture and massive demand of tilapia production in developing countriesin 1988, the Genetically Improved Farmed Tilapia (GIFT) project was initiated. The aim of this project was to genetically improve growth performance and adaptability to a wide range of environments through a systematic breeding programme. The founding population of GIFT comprised wild Nile tilapia from Africa (Egypt, Ghana, Kenya and Senegal) farmed Nile tilapia from Israel, Singapore, Taiwan and Thailand. . After 5 generations of selection, the growth performance of the GIFT strain was 80 % higher than the base population which indicated the suitability of selective breeding to improve the production performance of Nile tilapia (Puttaraksa 2004). To this date, the GIFT tilapia has been disseminated to 16 countries and is used in both small-scale and commercial aquaculture systems.

According to FAO , tilapias are the most popular aquaculture species group farmed in 127 countries in 2017 (Cai et al 2019). The major tilapia producer is China which accounts for 27 % of the world aquaculture production followed by Indonesia (22 %), Egypt (16 %), Bangladesh (5.7 %), Brazil (4.9 %), the Philippines (4.9 %) and Viet Nam (4.0 %). Among the 18 tilapia species cultured, Nile tilapia is the

dominant species in the group with 4.1 million tonnes of the total production (5.88 Mt) in 2017. In terms of production quantity, tilapia is the second only after cyprinid species (5.88 Mt and 28.3 Mt respectively) (Table. 1.1). However, the value of tilapia only accounts for USD 11 million or 4.42 % of world fish production value (Table. 1.1), reflecting the low value of this species which is mainly destined for local consumption.

Table 1.1 World Aquaculture production in 2017 (Cai et al 2019)

Species group	Number of ASFIS species items in the group farmed in global aquaculture	Number of countries farming the species group	World aquaculture production quantity of the species group (live weight; tonnes)	Share of world aquaculture production quantity of all species (%)	World production value of the species group (farmgate; USD 1,000)	Share of world production value of all species (%)
Carps, barbels and other cyprinids	38	92	28,345,338	25.32	61,437,284	24.62
Tilapias and other cichlids	18	127	5,880,586	5.25	11,031,140	4.42
Oysters	12	44	5,710,522	5.1	6,788,868	2.72
Clams, cockles, arkshells	29	21	5,658,458	5.05	9,779,660	3.92
Catfishes	27	86	5,518,878	4.93	10,569,972	4.24
Marine shrimps and prawns	14	60	5,511,914	4.92	34,220,879	13.71
Salmons, trouts, smelts	20	83	3,476,845	3.11	22,310,102	8.94
Other species	n.a.	n.a.	18,357,140	16.4	66,781,214	26.76

In Thailand, Nile tilapia was first introduced as a gift from the emperor Akihito of Japan to King Rama IX of Thailand in 1966. It was initially cultured from only 50 fish and bred by the department of fishery before being distributed to people in rural areas as a protein source. Due to the robustness of tilapia, the species easily gained popularity. However, decreased survival rate and reduced growth rate appeared after several breeding generations. Therefore, a new strain of Nile tilapia (*O. niloticus*), called "Chitralada tilapia", which had higher growth rate and less mortality of fry than the wild type was developed by selective breeding (Department of Fishery, ThailandChitralada tilapia has been continuously developed to improve strain robustness and market traits in order to service fish farms and market demand. Besides, Chitralada tilapia was one of the selected strain used in genetically improved farmed tilapia (GIFT). Production data from Thailand in 2017, showed that around 200,000

tonnes of Nile tilapia were produced with accounted for 52 % of total freshwater fish produced (Fig. 1.2) and 95 % of its production was consumed within country (Department of fishery, 2019). Thus, tilapia farming in Thailand has the possibility to expand in the further due to increasing of tilapia demand globally.

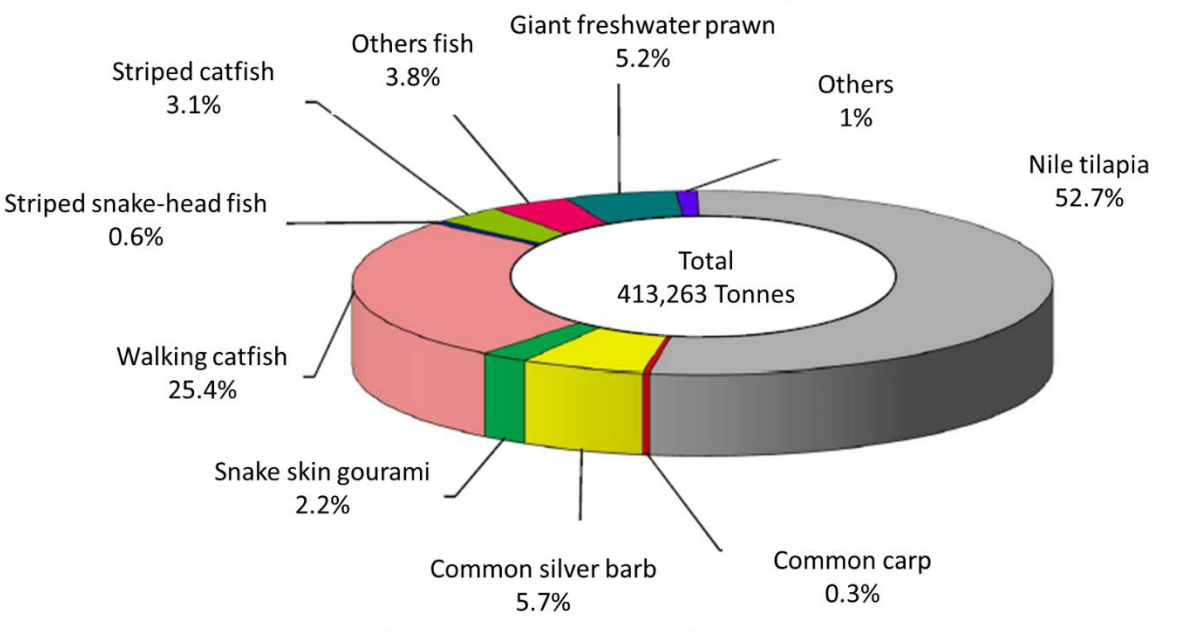


Figure 1.2 Top 10 species grouped by aquaculture production valume in Thailand in 2017 (Modification from Department of fishery, 2019)

1.1.2 Tilapia farming process and systems

The production of tilapia consists of 3 main stages; spawning and hatchery stage, nursery stage, and grow-out stage. The spawning and hatchery stages includes spawning of the brood fish and fertilization by male, then collect the fertilized eggs from the mouth brood female and incubate the eggs until hatching occurs and a new stock of fry is produced. In this stage, it is possible to produce all-male fry by treating the fry with Methyl testosterone before fry are transferred to the nursery stage to produce the fingerings (Rocky 2005). The nursey stage takes around 1-2 months from which fry of round 2-3 cm grow to 7-10 cm (fingerlings). The final stage, known as grow-out stage, includes growing the fingerlings till reach marketable size (0.5-1 Kg). The grow out stage, takes between 3-6 months depending on farming environment and food supply (Pongthana 2010). The example of Nile tilapia production on farm is shown in Figure 1.3

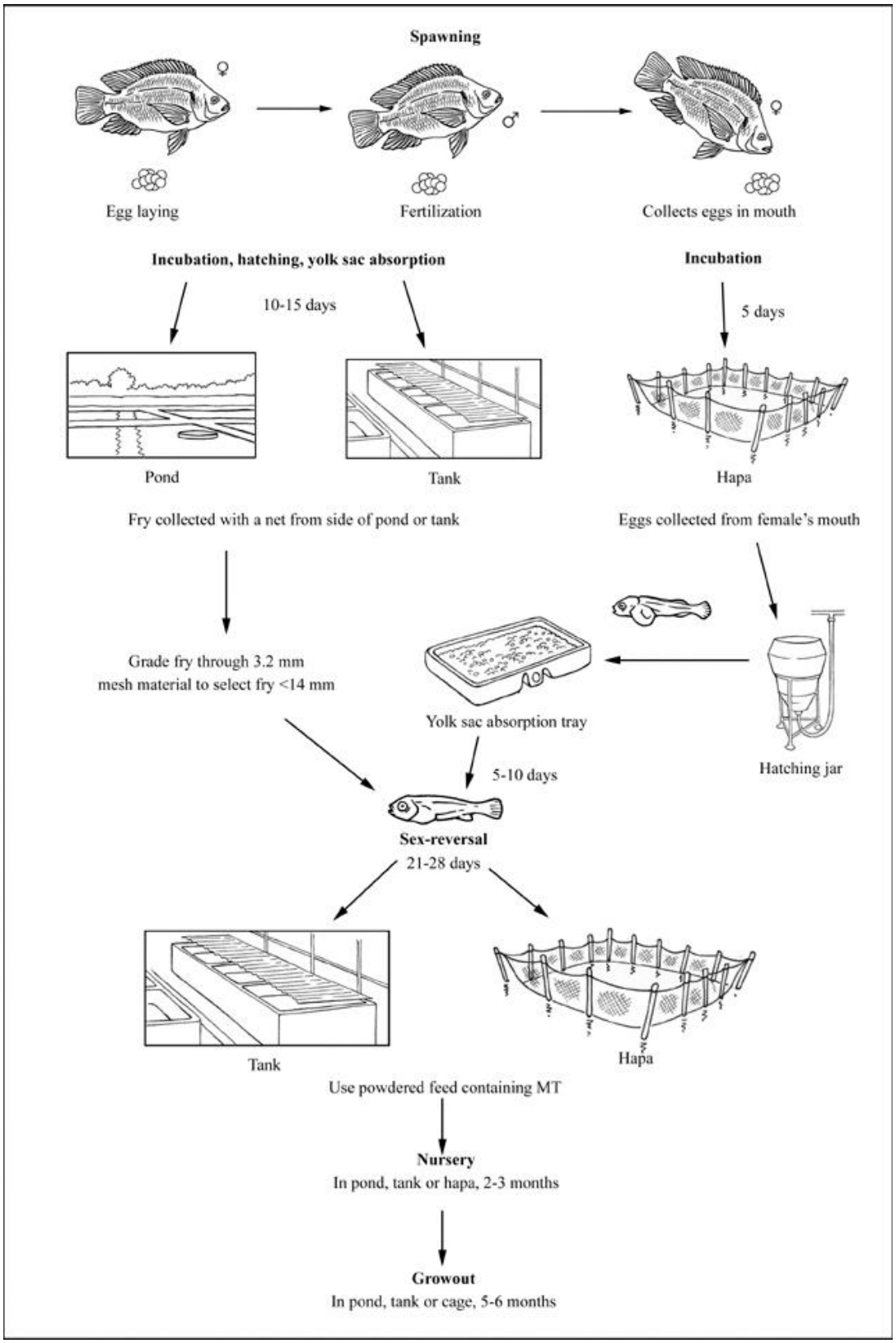


Figure 1.3 The production cycle of Nile tilapia. MT: Methyl testosterone. The figure taken from Rocky, 2005

In general, Tilapia farming can be farmed in extensive, semi-intensive and intensive systems. In the 1960s, tilapia was introduced to developing countries in order to improve food security and reduce poverty of rural populations; therefore, farmers were local people with limited in education, technology and finance. The extensive culture can be done in rivers, irrigation canals or lakes, using food supply from natural sources that reduce costs during production processes. However, it is complicated to control growth rate due to food supply depending on natural resources, thus good water quality is required in order to increase growth rate and decrease mortality. Even though its impact is difficult to measure, extensive tilapia culture is appropriate for household consumption and supports local demand which can raise family nutrition and living standards (El-Sayed 2006)

Semi-intensive farming of tilapia mainly done in ponds has been practiced in many developing countries, particularly in South-east Asia, using natural sources, through pond fertilization and supplementary feed (El-Sayed 2006). This culture system aims at increasing production yield while minimising production cost by limiting the quantity of commercial feeds, which can account for 50% of costs (El-Sayed 2008). In this system, fish feed from the food-web within the pond and are also given supplementary feed. Semi-intensive culture of tilapia can be practiced in a monoculture, polyculture or integrated agriculture-aquaculture systems (IAAS). In South-east Asia, tilapia is typically cultured with other fresh-water fish species in ponds that are integrated with agriculture and animal farming, particularly in Indonesia, Thailand, Vietnam, Cambodia and Myanmar (Gupta and Acosta 2004). This system seems suitable and profitable as most necessary inputs are available to the farmer such as rice bran, crop by-products and manure (El-Sayed 2006). Beside, tilapia-shrimp polyculture appears to be an effective way to control disease in shrimp farms as it maintains a stable plankton environment and increases shrimp survival (Cruz et al 2008; Wang and Lu 2015). The tilapia-shrimp polyculture has been applied in many countries such as Brazil, Mexico, and the Philippines (Wang and Lu 2015).

However, the increased demand of tilapia across the world and the competition between agricultural products has shifted tilapia culture systems from extensive and semi-intensive to intensive which can support larger-scale commercial production. Intensive farming systems are designed for growing fish at high stocking densities in ponds, tanks, raceways, cages or recirculating systems. The intensive culture of tilapia is an attractive commercial alternative due to the tolerance of tilapia to high stocking densities and are also tolerant to stress and handling (El-Sayed 2006). In terms of recirculating aquaculture systems (RAS), a critical role is water exchange, which allows to maintain oxygen levels

and remove waste. With very high stocking densities and controlled management systems, the production of tilapia can range from 100 to >500 Mt/ha/year (Beveridge and McAndrew 2012).

1.1.3 Diseases in Tilapia

In general, fish are surrounded by numerous of bacteria in their living environment. However, not all bacteria can cause disease and lead to severe outcomes such as mortality. This is particularly the case for tilapia which is known as being quite tolerant to cope with changing environmental conditions. However, the culture of Tilapia in intensive systems increases the possibility of disease outbreaks due to high stocking densities. Bacteria reported to cause disease in tilapia includes *Vibrio* spp, *Pseudomonas* spp, *Edwardisella tarda, Aeromonas hydrophila, Straptococcus agalactiae and Franciscella noatuenensis* (reviewed in Dong et al 2015). Furthermore, not only bacteria but also virus, parasites and fungi such as tilapia Lake virus (TiLV) (Eyngor et al 2014; Jansen et al 2018), *Icthyophthirius multifilis (*El-Galil et al 2012)*, Spironucleus* spp (Supamattaya et al 2012), *Trichodina* spp (Rameshkumar and Ravichandran 2010) and *Saprolegnia* spp (Zahran et al 2017) have been reported to affect tilapia. Of these, the pathogens associated with common disease outbreaks in tilapia are *Aeromonas hydrophila, Straptococcus agalactiae and Franciscella noatuenensis.*

1.1.3.1 Aeromonas hydrophila

A. hydrophila is a heterotrophic, Gram-negative, rod-shaped bacterium mostly found in warm climate regions (Fu et al 2014). It is the causative agent of motile aeromonad septicemia (MAS), which has caused high mortality rate and great economic loss in a wide range of aquatic species (both freshwater species and marine species) such as catfish (Hossain et al 2014), grass carp (Yang et al 2016), *Cyprinus carpio* (koi) (Wonglapsuwan et al 2015), pufferfish (Wang et al 2016), and Nile tilapia (Aly et al 2015). Moreover, it can cause disease in reptiles, amphibians, birds and humans, and it is also the cause of zoonotic diseases which spread between animals and humans (Daskalov 2006). In fish, *A. hydrophila* is an opportunistic pathogen, commonly found in water and also in the gastrointestinal tract. It will cause the disease when other factors are involved such as poor environmental conditions, handling stress and lack of nutrition (Swann & White 1991).

The clinical signs of infected fish can be observed at different levels from an acute mortality in healthy fish to abnormal behaviour such as lack of appetite and swimming abnormality The clinical signs of motile aeromonad septicemia include skin ulcerations ranging from surface to deep lesions in the skin, which are typically surrounded by a bright rim of red tissue (Wonglapsuwan et al 2015). Other clinical signs include distention of the abdomen, swelling of tissues, necrosis and hemorrhagic septicemia (Aly et al 2015). The consequences of a motile aeromonad septicemia outbreak can have devastating effects on the aquaculture production because it induces high mortality, reduction of growth due to the lack of appetite and it also leaves the fist unmarketable even after the disease is controlled. Besides, it is difficult to disinfect when a virulent strain of *A. hydrophila* becomes endemic in the culture environment.

1.1.3.2 Streptococcus agalactiae

Streptococcus agalactiae is a heterotrophic and beta-hemolytic Gram-positive bacteria which causes severe morbidity and mortality in several fish species both in freshwater and saltwater (Zhao et al., 2015), particularly for tilapia. Disease outbreaks have been reported in many countries such as Indonesia (Lusiastuti et al 2014), China (Li et al 2014), Malaysia (Firdaus et al 2013), Thailand (Jantrakajorn et al 2014) and Brazil (Mian et al 2009). Besides, infection by *Streptococcus* species can cause diseases in human newborns and domestic animals such as dogs, cows, horses and guinea pigs (Johri et al 2006; Mian et al 2009). For tilapia, the emergence of this disease usually occurs in high temperature periods, which causes tilapia to be more susceptible to streptococcosis (Fu et al 2014). The study of Zhao et al (2015) found that mortality of infected Nile tilapia became more severe at higher temperatures. Mortality reached 50% and 70% at 25 °C and 30 °C respectively, whereas no mortality was observed at 20 °C after intraperitoneal injection with *S. agalactiae.* Moreover, the disease tends to be more severe when Red tilapia are larger than 20 g. (Hernández et al 2009; Jantrakajorn et al 2014; Jiménez et al 2011).

Diseased tilapia typically shows acute clinical signs and high mortality usually occurs within a short period of time, approximately 10 days (Y. Li et al 2014). Jantrakajorn et al (2014) described the clinical signs of infected tilapiasuch as anorexia and abnormal swimming (e.g. spiral motion near the water surface or staying at the bottom of the pond). Other external signs reported include dark skin coloration with generalized hemorrhagic areas on the body surface, particularly at the mouth, operculum, fins and anus, skin ulceration and exophthalmia with corneal opacity. Moreover,

abdominal swelling and hemorrhaging of internal organs such as brain, heart, liver, stomach, intestine and gonads can be found. Finally, when the disease has been controlled, the surviving fish are unacceptable for marketing due to their physical appearance (hemorrhagic skin), low flesh quality and shorter shelf life (Jantrakajorn et al 2014).

1.1.3.3 Franciscella noatuenensis

In recent years, *Franciscella noatuenensis* has been reported as a serious emergent pathogen of various fish species, both farmed and wild, worldwide. *F. noatuenensis* can cause high mortalities and severe economic losses in species such as Atlantic cod (Gadus morhua), Atlantic salmon (*Salmo salar*), hybrid striped bass (*Morone chrysops* x *Morone saxatilis*) and tilapias (Colquhoun & Duodu 2011). This organism is a non-motile, Gram negative coccobacillus and it is classified as a facultative intracellular bacteria that can live in both fish cells and the environment (Soto et al 2013). There are two sup-species of *Franciscella noatuenensis* which can cause francisellosis in fish; *F. noatunensis* subsp. *orientalis* causes the disease in warm water fish species, whereas *F. noatunensis* subsp. *noatunensis* causes the disease in cold water fish species (Colquhoun & Duodu 2011). However, there are no published reports about zoonotic potential amongst these two sup-species of *Franciscella*. In tilapia, francisellosis is highly infective, only 23 colony forming units (CFU) can cause mortality in fingerlings, besides the disease can manifest itself as an acute disease, with few clinical signs and high mortality rates, or as a sub-acute to chronic infection with non-specific clinical signs and low mortality rates (Soto et al 2009).

Infected fish might show abnormal swimming (erratic swimming or stay at the surface of water), and they may be anemic and exophthalmic (Soto et al 2009), which are non-specific clinical signs. However, when examining internal organs, diseased fish present obvious clinical signs particularly in the spleen and kidney which are enlarged and contain white nodules (Soto et al 2009). These nodules are known as granulomas and contain numerous coccobacillus. According to Soto et al (2015), temperature plays an important role in the development of francisellosis in Nile tilapia *Francisella noatunensis*-challenged fish at 25 °C and 30 °C showed higher mortality specially in fish challenged at 25 °C than at 30 °C. In addition, increasing water temperature from 25 °C to 30 °C can prevent the development of clinical signs and mortality in challenged fish. These results suggest that *Francisella* tends to have a serious impact on tilapia when the water temperature decreases.

1.1.3.4 Disease development and control

The development and severity of disease within fish populations depends on the complexity of host-pathogen interaction and environmental factors. The virulence of a pathogen is one of the importance factors that determines the ability of the pathogen to cause disease. Different strains of a pathogen may show different severity and clinical signs when infecting the same host. The immune condition represents the resistance level of fish to pathogen. It is the result of the genetic composition of individuals and may be the result of their previous infection which can activate their immune response to a specific pathogen. However, poor water quality such as temperature, pH and dissolved gases can contribute to stress in fish: as the fish completely rely on water, they tend to be more susceptible to the disease. Particularly, changing of water temperature can cause the emergence of disease in tilapia such as streptococcosis (Fu et al 2014) and francisellosis (Soto et al 2015). Furthermore, poor nutrition status, handling and transport also can induce stress and lead to disease outbreaks in fish populations.

When the disease is manifested in the population, chemotherapeutic agents have been used to disinfect and control the disease. Antibiotics that have been used to control a motile aeromonad septicemia include penicillin, ampicillin, carbenicillin, erythromycin, streptomycin and clindamycin (Jones & Wilcox 1995), whereas amoxicillin, ampicillin, oxytetracycline, enrofloxacin, erythromycin and sulphamethoxazole/trimethoprim have been used against streptococcal pathogens (Jantrakajorn et al., 2014). To treat an infection of *Francisella*, it is considered to use Florfenicol, Flumequine, Oxolinic acid, Oxytet/tetracycline, Amoxicillin, Chloramphenicol, Erythromycin and Pencillin G (Colquhoun & Duodu 2011). However, there are growing concerns of side-effects of using those reagents as they can accumulate in fish and the environment which can cause potential food hazards, and they increase the development of antimicrobial resistance (AMR) by gene transfer (Aly et al 2015).

Several studies have examined AMR such as the study by Tipmongkolsilp et al (2012), who found that 55 isolates of *Aeromonas hydrophila* from infected Nile tilapia in Thailand were resistant to at least one drug from 11 tested and more than 50% of all isolates were resistant to other five drugs tested. In Malaysia, 21 isolates of *A. hydophila* from infected blue tilapia were resistant to ampicillin and most of the isolates showed multiple-drug resistance (Son et al., 1997). Finally, Jantrakajorn et al (2014) found that approximately 24 isolates of *S. agalactiae* from Nile tilapia in Thailand were resistant to at least three antimicrobials.

Therefore, alternative disease control methods are required. The improvement of health and prevention of stress by feeding through proper nutrition or supplementary feed, avoiding unnecessary handling and extreme environmental conditions are necessary to reduce stress and diseases in fish farms. For example, the use of tapioca-based biofloc treatment with *Labeo rohita* fingerlings enhanced growth rate and non-specific immune response against *A. hydrophila* (Verma et al 2016). Moreover, stimulation of the immune system by vaccination is seen to be an effective way. Many bacterial vaccines have been developed such as a vaccine against *A. hydrophila* in Nile tilapia (Aly et al., 2015) and common carp (*Cyprinus carpio*) (Poobalane et al., 2010), oral vaccine against streptococcosis in Nile tilapia (Firdaus et al 2013) and *Francisella noatunensis* subsp*. orientalis* vaccine in Nile tilapia (Soto et al 2013). However, vaccination has some limitations. The price might not be affordable for fish producers and it might be difficult to apply in a large population. Selective breeding for disease resistance is another effective way to control and reduce bacterial diseases. Several studies have examined the association of allelic variation of particular genes to bacteriaL disease resistance in tilapia, in order to facilitate selective breeding (Fu et al., 2014). However, disease resistance is a new trait in tilapia breeding programmes which require further study in relevant to disease resistance trait or host-pathogen interaction.

1.2 Fish immune system

The immune system is a biological defence system of a host against foreign agents such as viruses, bacteria and parasites. Like other vertebrates, fish immune system can be divided into innate and adaptive immune systems. In simple terms, the innate immune system is the first line of defence in which a rapid response to a non-specific pathogen is performed. This system is driven by recognition of highly conserved, structural or interior, molecules present in pathogens referred to as Pathogen Associated Molecular Patterns (PAMPs) that trigger an initial response. The adaptive immune system, which has a specific response to the pathogen is activated subsequently in a timeframe ranging from minutes to weeks (Fig 1.4). In fish, the immune system depends on their environment, their poikilothermic nature, and the number of immunocompetent cells is dependent on body weight rather than the age of the fish (Tort et al 2003).

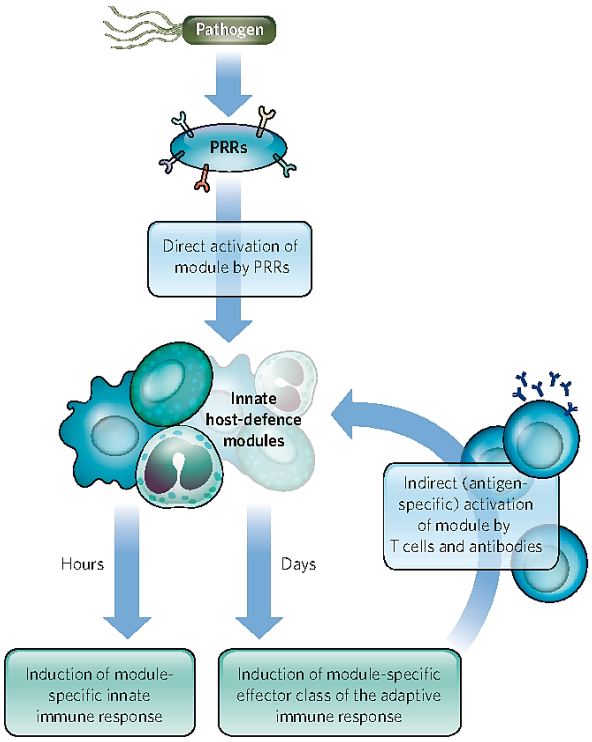


Figure 1.4 Activation of host-defence mechanisms. The defence mechanisms of the host are directly induced by an engagement of PRR-PAMP or indirectly induced by T cell/antibodies. The PAMPs are recognised by specific PRR that trigger an innate immune response in an early stage of infection and can induce adaptive immune response after a period of time (Medzhitov 2007)

The central organs which control the main functions of both cellular and humoral immune responses in fish are similar to all vertebrates. These are the thymus, kidney and spleen which are considered the largest lymphoid organs in teleost (Uribe et al 2011; Zapata et al 2006). The thymus, produces T lymphocytes which are involved in the stimulation of phagocytosis and production of antibodies generated by B lymphocytes (Kiron 2012; Zapata and Amemiya 2000). In fish, it is believed that the development of the thymus is more dependent on hormonal cycles and seasonal variations than on the age (Rauta et al 2012). The kidney in teleost is comparable to bone marrow in vertebrates as it is the largest site of haematopoiesis (Uribe et al 2011). The kidney in fish is a Y shaped organ and placed along the body axis. The head kidney is a main part of the kidney and includes key regulatory functions such as antibody processing and formation of immunoglobulin M (IgM). Furthermore, it is a central organ for immune-endocrine interaction and neuro-immuno-endocrine connections (Rauta et al 2012). Whereas, the spleen functions as a secondary immune organ after the kidney for the innate and the acquired immune systems, involved in haematopoiesis, antibody production and antigen

degradation (Kiron 2012). In fish, the size of the spleen is used as a measurable parameter to observe the immune response against parasite infections (Lefebvre et al 2004; Rauta et al 2012).

1.2.1 Innate immune system

The innate immune system is a fundamental defense mechanism of fish and all known animal life which is characterised by non-specific responses and recognition of danger signals such as pathogen-associated molecular patterns of bacteria, fungal glycoproteins, lipopolysaccharides and intracellular components released through injury or infection (Magnadóttir 2006). This system is inducible by these stimulatory factors and responds to such factors immediately however is thought to be a short-term response. Besides, the innate immune system plays a role in activating the adaptive immune system. It is commonly divided into three categories; physical barriers, cellular and humoral components.

Physical barriers

Physical barriers (e.g. skin mucus and gill) are considered the primary barrier to infection. Skin is a layer interface that separates the internal and external environments, providing physical and chemical protection in connection with the mucus to prevent the infection of microorganisms and for osmotic balance (Parra et al 2015) . The external surface or skin of most fish species is covered by mucus, particularly in freshwater species. Fish significantly increase the production of mucus when they are confronted by stress (Tort et al 2003). Whereas gill consists of gill epithelium, glycocalyx layer and mucus layer. Gills are a multifunctional organ involved in gas and ion exchange, osmotic balance, hormone production and ammonia excretion. Besides, Gill is major organ related in antibody secreting cell production after immersion immunization as it was found an accumulation of lymphocytes at the base of the gill filament in salmonid fish (Haugaruall et al 2008; Secombes 2012).

Cellular immune response

The cellular innate immune response has diverse functions ranging from phagocytic cells that engulf and degrade particles, mononuclear cells that produce and secrete signals such as cytokines and chemokines to stimulate cell migration to macrophages that present antigen (Reyes-Cerpa et al 2013). As an example, phagocytosis is an essential mechanism that defends against pathogen infection by

enveloping the invading particles in a receptor-specific manner on the cell surface (i.e. macrophages or monocytes, granulocytes or neutrophils and non-specific cytotoxic cells). There are two functions of phagocyte killing; intracellular killing (within the phagocytes) and extracellular killing (outside the phagocytes).

Intracellular killing is divided into two types. The first is an oxygen-dependent process. This method produces toxic oxygen compounds to kill the pathogen by using superoxide dismutase to convert superoxide to hydrogen peroxide and singlet oxygen, resulting in hydroxyl radicals which assist in the killing process (Mak and Saunders 2005) or by using myeloperoxidase to produce hypochlorite (hydrogen peroxide and chloride), which is toxic to bacteria. The second is an oxygen-independent process, grouped into four types; 1.) Use electrically charged proteins to damage the microbial membrane, 2.) Use lysozyme to breakdown the microbial cell wall, 3.) Use lactoferrins from neutrophil granules to remove essential ion from bacteria and 4.) Use proteases and hydrolytic enzymes to digest protein in the bacteria (Delves et al 2011). On the other hand, extracellular killing relies on the production of nitric oxide which is toxic to bacteria. Nitric oxide is secreted by natural killer cells, B cells, T-cells, monocytes, macrophages or dendritic cells through stimulation with interferon gamma (IFNγ) (Schroder et al 2004).

The humoral immune response

The humoral immune response has diverse components that inhibit adherence and colonisation of microorganisms and is present in serum, mucus, skin, gills, and intestine (Kiron, 2012). It includes various antimicrobial agents such as trypsin, lysozyme, natural antibodies, complement factors and other lytic factors (Alexander and Ingram 1992; Kiron 2012). For example; Lysozyme is a lytic enzyme, found in mucus, lymphoid tissue, plasma and other fluids (Rauta et al 2012). It is a hydrolytic enzyme that cleaves the beta-1, 4 glycosidic bond between N-acetylmuramic and N-acetylglucosamine in peptidoglycan of the bacterial cell membrane in gram-positive bacteria resulting in bacterial cell lysis (Magnadóttir 2006). Gram-negative bacteria can also be lysed by lysozyme but after the outer membrane has been destroyed by complement (Saurabh and Sahoo 2008). In fish, lysozyme is synthesised in the liver as well as in extrahepatic tissues (Bayne and Gerwick 2001; Kiron 2012). The lysozyme response has been used as a parameter in immune defence because its potency varies in a species-specific manner, it is rapidly induced and also responds to many stress conditions (Demers and Bayne 1997; Rotllant and Tort 1997; Tort et al 2003).

The complement system has a primary role in the innate immune response and links between both innate and adaptive immunity in fish. The complement is a system of serum proteins which are central in many defence mechanisms, comprised of several components such as C3, C7, C4, C5 and factor B (Kiron, 2012). In bony fish, there are two pathways of activation; classical complement pathway (CCP) and alternative complement pathway (ACP) (Rauta et al 2012). The classical complement plays an important role in the killing of pathogens through opsonization and the activation of phagocytes. The alternative complement pathway is activated by lipopolysaccharide in the outer membrane of Gram-negative bacteria and results in lysis of the bacterial cells (Rauta et al 2012).

Lectins are carbohydrate-binding proteins that act as opsonins to promote phagocytosis or the complement cascade activator (Magnadóttir et al 2005). There are 2 major types of lectin, S-type and C-type, classified according to sequence data and biological function. The S-type lectins actas intracellular protein and extracellular protein with no disulphide bonds and recognize predominantly galactose. Whereas, the C-type lectins are extracellular proteins unrelated to the S-type; consist of a large superfamily of membrane that share a disulphide-rich Ca^{2+} binding CRD (non-catalytic carbohydrate-recognition domain). C-type lectins have an important role in the immune system as they act as membrane receptor proteins, some of them are predominant effectors in innate immune system and other involve leukocyte trafficking and cell-cell interaction (reviewed in Ewart et al 2001).

Acute phase proteins (APP) are proteins which cooperate in the systemic response to inflammation: most notable are the C-reactive proteins (CRP), serum amyloid P (SAP), and serum amyloid A (SAA). They are present in diverse defence-related activities such as limiting the dispersal of infectious agents, inactivation of proteases, killing of microbes, repair of tissue damage, and restoration of the healthy state (Bayne and Gerwick 2001; Rauta et al 2012). APP operates the activation of the classical complement pathway, phagocytosis and is also related to removal of apoptotic cells (Rauta et al 2012), and their response depends on the level of infection and injury.

1.2.2 Adaptive immune system

Acquired/adaptive/specific immunity is a mechanism which is mainly activated by the innate immune system however some cells of the adaptive system, B lymphocytes, are capable of independent recognition and activation. The adaptive immune system has the unique ability to recognize pathogens encountered in previous infections, this is known as immunological memory. This system has a relatively slow response and requires a period of time to develop however once developed the response can persist for a long time and is highly effective against pathogens. The key cells of the adaptive immune response are lymphocytes: B lymphocytes mediate antibody responses (humoral immune response) and T lymphocytes mediate cell-mediated immune responses (Secombes & Wang 2012).

In general, B cells activate and secrete antibodies when B cell receptors recognize foreign antigens. The antibodies circulate throught the body via the bloodstream and penetrate all body fluid compartments where they bind to the antigen (Secombes & Wang 2012). Antibodies are able to inactivate microbes or viruses by blocking the binding ability of pathogens to receptors on host cells. Antibody binding to pathogen-surfaces also provides a signal for cell-mediated destruction of infected cells for example through Fc-receptor systems on macrophages (Secombes & Wang 2012). T cells directly respond to a specific antigen, which has been presented, by MHC systems, on the surface of the host cells. T cells eliminate pathogen-infected cells and cooperate with the innate immune system to defend against such pathogens.

The humoral adaptive immune response

B lymphocytes are a key feature of the humoral adaptive immune response performing various roles including memory B cells and forming antibody-producing plasma cells for antibody production. B cells can directly interact with antigens through the surface B cell receptor complex (BCR), antigen is then internalized and degraded for presentation. MHC II presentation acts as a signal for T helper cells which activate and support B cell differentiation into plasma B cells and memory B cells. The plasma B cells differentiate into antibody-producing cells directly acting against the current infection whereas a portion of B cells specific to the pathogen remain in the lymph nodes forming long-lasting memory against specific pathogens (Mak & Saunders 2005).

Immunoglobulins (Ig) are specialised proteins, mainly produced by B cells. Structurally, each arm of the Ig molecule has an antigen-specific binding site to a specific epitope on the antigen. A typical Ig, such as IgG in mammals, is composed of two heavy chains (H) and two light chains (L). Each chain contains different segments; the heavy chain has three segments (V, D and J) and the light chain has two segments (V and J). Antibodies can be categorized into different isotypes based on the structure of their heavy chain. In fish, there are three isotypes which have been identified in almost all studied species; IgM, IgD and IgT. IgM is expressed as a tetramer and is the predominant Ig isotype in blood and serum of fishes. Both IgM and IgT are found at early stages of fish development and a significant increase of IgT during development is also noted suggesting that both isotypes play an important role in protecting fish larvae (Secombes & Wang 2012).

The cellular adaptive immune response

T cells or T lymphocytes are a group of lymphocytes which have a major role in cell-mediated immunity. They are categorized into T helper cells (Th cell), cytotoxic T-cells (CTLs), memory T-cells, regulatory T-cells (T_{REG} cell), natural killer T-cells (NKT cell) and mucosal associated invariant T-cells (MAIT) (Mak & Saunders 2005). T helper cells are key to the cell-mediated immune response as they are involved in the activation and differentiation of B cells, and the activation of cytotoxic T-cells and macrophages. T cell responses to infection are antigen-dependent; intracellular and extracellular. Intracellular antigens originate within the cell and are mainly presented through the MHC class 1 system e.g. virus and cancer cells, these are recognized by CD8 cytotoxic T-cells (CD8 CTLs). Whereas the extracellular antigens are found outside the cells, e.g. bacteria and parasites, and are presented through the MHC class II system on professional antigen presenting cells (APCs) such as dendritic cells driving CD4 T cell responses. T helper cells recognize the antigen presented by MHCII through a combination of T cell receptors (TCR) and CD4 molecules on their surface hence the name CD4 T cells. There are also several other co-stimulatory cell surface molecules involved such as CD28 that recognizes CD80 on the APC. When T cells are activated they then release cytokines to regulate and promote the immune response such as the differentiation of B cells. Interleukin-2, a critical cytokine in T cell regulation, stimuates the production of T cells and activates T cell differentiation into the formation of memory and effector T cells (Th1 and Th2). Th1 cells activate cytotoxic T cells and stimulate macrophages, while Th2 activate B cells and humoral immunity (Mak & Saunders 2005). In the case of CD8 CTLs, the Th1 response is driven by interleukin 2 promoting cellular proliferation to enhance the CD8 response. The mechanisms of killing infected cells by CD8 CTLs include cytotoxic granules and Fas pathway. Cytotoxic granules,

contain perforin and granzymes that are released on contact with the target cell. Perforin forms a pore in the membrane of the target cell allowing granzymes to enter. Granzymes denature the production of viral protein resulting in destruction of the infected cell. The Fas pathway when activated through expression of cell-surface Fas ligand induce activation of the caspase cascade upon ligand binding activating apoptosis in the target cell (Mak & Saunders 2005).

1.3 PAMP-PRR recognition and the immune response

The rapid non-specific target response of the host innate immune system is activated by Pathogen Associated Molecular Patterns (PAMPs), which are endogenous components structure on the surface or inside the pathogen. Medzhitov (2007) mentioned that PAMPs are well recognised by host immunity because they are essential in microbe physiology which is conserved throughout evolution, thus limiting the ability of the microbe to avoid host defence mechanisms. Important PAMPs include lipopolysaccharides (LPSs) and peptidoglycans (PGNs) found in the bacterial cell wall, -glucans of fungi, and viral nucleic acids. PAMPs such as LPS, PGN and Poly (I:C) (artificial dsRNA) are commonly used in comparative immunology studies. LPS is an important component found in Gram-negative bacteria. LPS activates innate immunity cascades by binding to LPS binding protein in the host bloodstream during acute infection (reviewed in Akira et al 2006). PGN is also an important bacterial cell wall component found in both Gram-positive and Gram-negative bacteria consisting of glycan chains cross-linked with short peptides (Vollmer et al 2008). PGN is a thin layer covering gram-negative bacteria whereas it is many times thicker in gram-positive bacteria. PGN is also an effective immunostimulant driving macrophage activation, cytokine production, autoimmunity and antimicrobial peptide production (Boneca 2005).

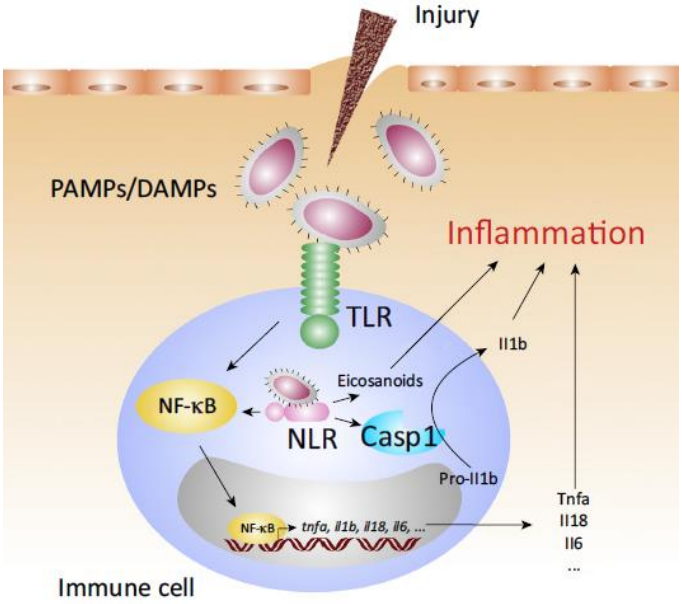


Figure 1.5 A key step of the classical inflammation pathway PAMPs are recognised by TLR that reside at the cell surface and trigger pro-inflammatory cytokines transcription via NF-γB, while NLRs are intracellular receptors that trigger inflammation via NF-γB and caspase (Espin-Palazon et al 2018).

PAMPs are recognised by host innate immunity mediator called Pattern Recognition Receptors (PRRs) which are germline encoded and broadly specific to conserved components or PAMPs of microorganism (Medzhitov and Janeway Jr 1997). There are several classes of PRR; Toll-like receptors (TLRs), NOD-like receptor (NLRs), C-type lectin receptor (CLRs), RIG-like Receptors (RLRs) and peptidoglycan recognition proteins (PGRPs). These receptors are located at the different subcellular location of the cells depending on their recognition functions where NLRs and RLRs are present in the cytoplasm, and TLRs are present at both the cell surface and the endosome (Mogensen 2009). The function and structure of PRRs are well conserved across the vertebrates (Boltana et al 2011). Among several classes of PRR, TLRs are the best characterised, they are receptors that recognise viral nucleic acid, LPS and lipoteichoic acid of bacteria. In fish, there are 18 TLRs however, the presence or absence of specific TLRs depends on the species (Zhang et al 2013). The function of TLR members has been relatively characterised; TLR2 mainly recognises bacterial production localised at the cell surface, while TLR3, TLR8 and TLR9 are expressed in the intracellular compartment; endosomes and lysosomes. TLR3 recognises dsRNA virus and dsRNA release from dead/necrotic cells. TLR8 is sensitive to G/U-rich ssRNA virus in endosome and TLR9 is a receptor for bacterial and viral DNA by recognising unmethylated CpG DNA (Seth et al 2006). TLR4 is a cell surface receptor that has been reported to recognise bacterial LPS

in mammals (reviewed in Kawai and Akira 2010) but absent in all fish except zebrafish (Sepulcre et al 2009). NLRs are intracellular cytosolic receptors that are specific to bacterial molecules including PGN and MDP (Meylan et al 2006) and are involved in the antibacterial and antiviral responses, apoptosis and autoimmunity reviewed by Boltana et al (2011). In some cases, TLRs cooperate with NLRs to stimulate inflammation for example; TLRs promote gene expression and intracellular accumulation of interleukin 1-β (IL-1β) and IL-18 via nuclear factor kappaB (NF-κB). On the other hand, NLRs in the cytosolic compartment induces caspase-1 activity that promotes the production of pro-inflammatory cytokines, including IL-1β activity (Espin-Palazon et al 2018) (Fig.1.5). In terms of antiviral responses, RLR and TLR3-based recognition are critical. RLRs are cytosolic PRRs that sense different viral molecules and the RLR group is formed by MDA5 (melanoma differentiation-associated gene 5), RIG-I (retinoic acid-inducible gene I) and LGP 2 (laboratory of genetics and physiology 2). In a study of SAV-3 infection in Atlantic salmon (*Salmo salar*) it was demonstrated that the RLR and TLR pathways were fundamental to the antiviral response and both cooperatively use IRFs to induce IFN production (Xu et al 2016) (Fig. 1.6).

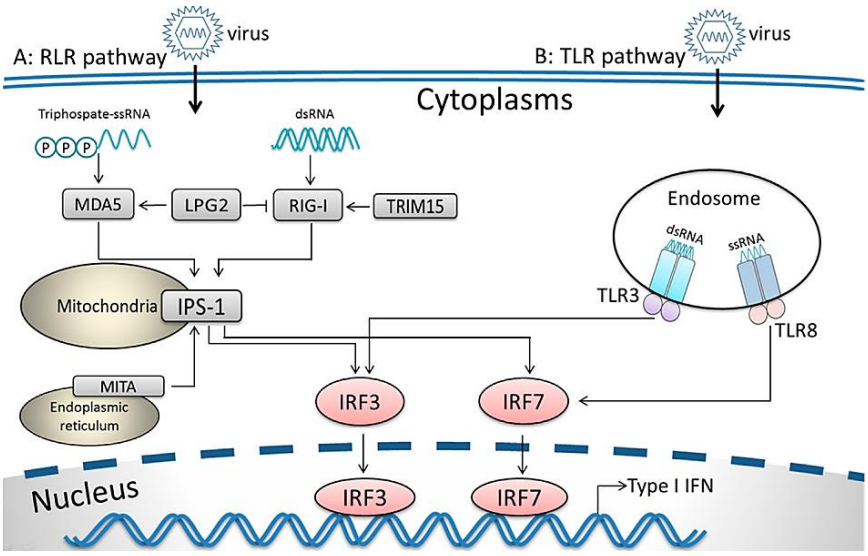


Figure 1.6 An antiviral response via the RLR pathway (A) and TLR pathway (B). Both pathways are intracellular receptors that specific to viral component and operate antiviral response (IFN) via IRFs (Xu et al 2016).

1.4 Macrophages

Macrophages are a critical cell type essential to the development of the immune response and are key in bridging both the innate immune and adaptive immune systems. In fish, macrophages are generated from hematopoietic stem cells located in the head kidney and then are released into the bloodstream as monocytes (Ribas et al 2008). The monocytes move along the vascular surface or attach to the epithelium before penetrating into tissues (Maslin et al 2005). This process known as trans-endothelial cell migration induces further cell differentiation and a transition from the monocyte phenotype to the mature macrophage. Macrophages are professional phagocytes that have a role in detecting and eliminating invading pathogens through PRR-based activation and can subsequently behave as antigen presenting cells (APC) that trigger the adaptive immune system. There are various receptors expressed on their surface including TLR, scavenger receptors, CLRs and complement receptors. It was found that a stimulation of fish macrophages *in vitro* with stimuli such as LPS, lipoteichoic acid (LTA) or peptidoglycan (PGN), flagellin and Poly I:C are able induce respiratory burst activity and production of oxygen radicals, phagocytosis and pro-inflammatory production (Secombes & Wang, 2012). Macrophages are a major source of immune-mediators including cytokines and prostaglandins which are key orchestrators of the immune response.

Cytokines are soluble proteins that are critical players in regulating the immune response by driven inflammatory signals which regulate the capacity of resident phagocytes and recruit phagocytic cells to destroy the invading pathogens. Cytokines also have an important role in regulating antigen presentation function of APC to initiate the adaptive immune response. Cytokines are classified into 2 groups; pro-inflammatory cytokines and anti-inflammatory cytokines. A major characteristic of cytokines is the ability to use cross-regulation to control the behaviour of the immune system through complex interacting networks. The pro-inflammatory cytokines e.g. TNF-α, IL-1β and IL-6 are mainly synthesized in ER of the macrophages and play as endogenous pyrogens to activate cell signaling cascades and trigger responses such as Th1 cell differentiation to induce inflammation and fever after infection (Duque and Descoteaux 2014). On the other hand, anti-inflammatory cytokines e.g. IL-10 and TGF-β have an antagonistic function to pro-inflammatory cytokines, as they have a role in suppression pro-inflammatory secretion to reduce inflammation and promote a healing stage (Fig. 1.7). TGF-β is a regulatory cytokine which involved in cell proliferation, differentiation, migration and apoptosis under physiological and pathological conditions and TGF-β also limit the proliferation of T cell by inhibiting

the production of IL-2 (Li et al 2006; Yang and Zhou 2008). Whereas, IL-10 is a key role in inhibition of cytokines synthesis which is mainly exerted against TNF-α, IL-1β, IL-6, GM-CSF and IFN-γ (reviewed in Fioranelli and Roccia 2014) and also directly inhibit reactive radical species, phagocytosis and antigen presenting process which indirectly limit function of Th cells (Piazzon et al 2015).

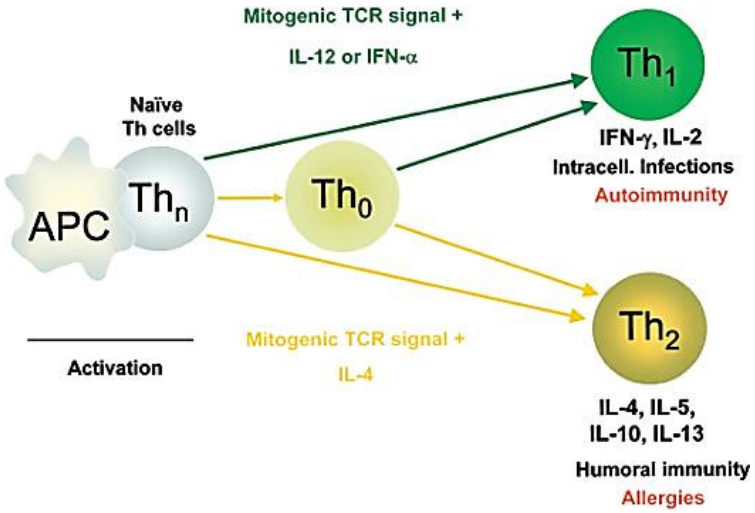


Figure 1.7 Th1 and Th2 differentiation; the naïve T-cells are developed to Th_0 cell by activation of APC before polarization into Th1 or Th2. In a present of microorganism, Th1 are induce by IL-12 or IFN-α to activate immunity against infection, whereas Th2 are induced upon a present of IL-4 to alter the response into the healing stage. TCR; t-cell receptor (Biedermann et al 2004).

Prostaglandins are regulators of the inflammatory response having both promotng and inhibiting actions. Prostaglandins are secreted from activated macrophages and modulate their function in an autocrine fashion (Niho et al 1998). Prostaglandins are comprised of several isoforms including PGE_2, PGI_2, PGD_2, $PGF_{2\alpha}$ and they are involved in many biological functions; blood pressure, gastrointestinal integrity and fertility (Ricciotti and FitzGerald 2011). Prostaglandin generation depends on the activity of cyclooxygenases (COXs) which have two isoforms; COX-1 and COX-2 (Dubois et al 1998). COX-1 is present in most cells and functions as a housekeeping gene that controls physiological processes (Dubois, 1998; Ricciotti and FitzGerald 2011) while COX-2 is important in prostanoid producer, it is highly upregulated upon activation of pro-inflammatory signals; LPS, IL-1β and TNF-α (Park,2006; Hamidzadeh;2017). PGE_2 has an important role in inflammation regulation as it was found that, upon TLR stimulation, PGE_2 suppresses IL-12 and TNF-α and partially suppresses IL-6, but induce IL-10 production in macrophages at the same time. Moreover, PGE_2 affect an induction of IL-17 leading to M2 macrophages development. In contrast, it reduce an expression of MHC II on antigen presenting

cells after their immigration to lymphoid tissue to suppress antigen presentation. In addition, intracellular parasites are able to stimulate the production PGE_2 to suppress inflammation and prevent cell necrosis for promoting a suitable environment for their survival (Reviewed in Hamidzadeh 2017).

In response to microbial infection or injury, macrophages have 2 distinct phenotypes with different functions, this is known as macrophage polarization; classical activated macrophages (M1) and alternatively activated macrophages (M2) have opposing functions in killing and healing. Wang et al (2014) reviewed the M1 phenotype in response to PAMPs and/or pro-inflammatory cytokines such as IFNγ, TNF or TLRs. M1 function is to promote pro-inflammatory cytokine secretion including TNFa, IL-1, IL-6, IL-12, Type I IFN, phagocytosis and antigen presentation that in turn drives Th1 activity and the acquisition of a strong antimicrobial response. In contrast, the M2 phenotype represents a healing/resolution function promoted by IL-4/13 and IL-10 activity post-infection. The M2 macrophage phenotype produces anti-inflammatory cytokines such as IL-10 and presents a low level of pro-inflammatory cytokines. This promotes the Th2-type response and is also involved in responses to parasites/chronic infection. M2-based functions include tissue remodelling and immune tolerance (Fig 1.8). Importantly, the polarization of macrophages is a highly dynamic process that can be reversed upon physiological and pathological conditions.

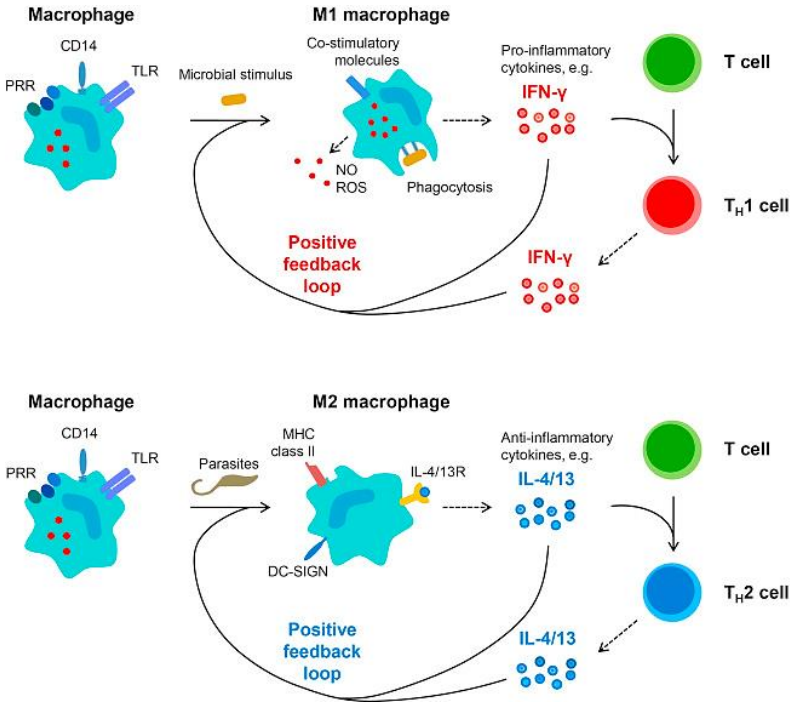


Figure 1.8 An overview of macrophages polarization and regulation of each phenotype (Wiegertjes et al 2016)

1.5 Aims of the study

Nile tilapia (*Oreochromis niloticus*) is one of the most important commercial fish species in the world and it is considered relatively tolerant to disease and different environmental conditions. Surprisingly, although many studies have addressed tilapia and diverse aspects of its aquaculture including growth rate, nutrition, culture techniques etc., there is very limited data available on molecular and cellular immunity. In order to support health and production management in tilapia farming, it is necessary to understand the immune response in order to develop health management and welfare strategies. Therefore, the objectives of this thesis were;-

- To develop a macrophage primary cell culture technique for Nile tilapia in order to further explore the molecular regulation of the immune response.
- To characterize the interaction between PAMPs and PRR in Nile tilapia in order to provide a knowledge platform for understanding the molecular regulation of the immune response, particularly inflammatory cytokines
- To detect intracellular parasites infection in the tropical aquarium and it infection in Nile tilapia by using macrophage primary cell culture and PCR-based method to increase efficiency and sensitivity of detection.
- To characterize the baseline transcription profiles of selected genes involved in immune response among different tissues of healthy tilapia grown in different culture conditions in order to examine the interaction between culture environment and strain of Tilapia with the aim to support tilapia fish farm management in the future.

Chapter 2

Development and characterisation of a Tilapia macrophage primary cell culture

2.1 Introduction

Nile tilapia (*Oreochromis niloticus*) is considered as one of the most important commercial fish in the world and surprisingly, there is limited knowledge available related to the molecular and cellular immune response. It is necessary to understand the immune system of fish, particularly the interaction between pathogen recognition receptors (PRR) and pathogen-associated molecular patterns (PAMPs) that operates the immune response, for both basic research and fish health management. Macrophages are an essential component of an immune response. They play a central role in various immunological reactions (Wang et al 2014) including the elimination of invading particles by phagocytosis, the regulation of inflammation by the production of inflammatory cytokines and prostaglandin E_2 and contribution of adaptive immunity as antigen-presenting cells.

Phagocytosis is a critical early event in response to invading pathogens by recognition and ingestion of particles larger than 0.5 μm into the phagosome. Discrimination between self and nonself molecules of phagocytes rely on a variety of receptors which recognize a target particle and initiate a signalling cascade that promotes phagocytosis (Rosales and Uribe-Querol 2017). Phagocytes can be categorized into 2 groups; professional phagocytes (monocytes/macrophages, and neutrophils) that mainly eliminate invading pathogens and activate adaptive immunity, while non-professional phagocytes (epithelial cells, fibroblasts, and dendritic cells) have a limited set of targets and a slow reaction but are essential in tissue remodelling and homeostasis by apoptotic cell clearance (Lim et al 2017).

Another critical function of macrophages is Prostaglandin E_2 production (PGE_2), which is a regulator of the inflammatory response having both promotion and inhibition actions, secreted from activated macrophages and modulates their function in an autocrine fashion (Niho et al 1998). Prostaglandins

are comprised of several isoforms including PGE_2, PGI_2, PGD_2, $PGF_{2\alpha}$ and they are involved in many biological functions; blood pressure, gastrointestinal integrity and fertility (Ricciotti and FitzGerald 2011). Prostaglandin generation depends on the activity of cyclooxygenases (COXs) which have two isoforms; COX-1 and COX-2 (Dubois et al 1998). COX-1 is present in most cells and functions as a housekeeping gene that controls physiological processes, while COX-2 is important in prostanoid producer in inflammation, activated by inflammatory stimuli, hormones and growth factors (Dubois, 1998; Ricciotti and FitzGerald 2011).

To understand the role of the immune system and interaction between pathogen and host, macrophages have been widely used as an *in vitro* model using cell culture techniques. In this study, a primary cell culture technique was used to develop macrophages primary cell culture for Nile tilapia. As primary cell culture is a useful technique to study functions of cells which are obtained from fresh tissue and a specific cell type enriched under proper and specific culture conditions. The primary cell culture has benefits to study cell populations when no cell line is available and can help to determine specific functions of the cell, which may be lost during secondary cell line development. However, the difficulty of performing primary cell culture is manifold including; the development of suitable conditions, which mimics the natural living environment of the cell, for cell growth and maintains its natural behaviour. It is important to highlight that the specific functions or the natural behaviour of cells can be changed or postponed dependent on the culture environment. Moreover, due to a limited life span of the cell, it requires a fresh tissue for cells isolation in every culture (Unchern 1999). Therefore, a proper tissue for cell isolation is another factor that should be concerned before doing primary cell culture. In terms of macrophages, the number of monocyte/macrophage cells depend on the tissue where they are derived. In teleost, the head kidney is the main hematopoietic tissue which produces erythrocytes, thrombocytes, lymphocytes, monocytes and granulocytes. The monocytes are released into the bloodstream and then develop into macrophages after they migrated into tissue (trans-endothelial migration). Thus, the common tissues that have been used for the isolation of monocyte/macrophage cells are blood, head kidney and spleen. Macrophage primary cell culture has been widely used as a model to study the interaction between PAMPs and PRRs in other fish species including rainbow trout (*Oncorhynchus mykiss*) (MacKenzie et al 2003, Hong et al 2003 and Costa et al 2011), European eel (*Anguilla Anguilla*) (Callol et al 2013), goldfish (*Carassius auratus*) (Grayfer et al 2011 and Haddad et al 2008), grass carp (*Ctenopharyngodon idella*) (Wang et al 2016) and Miiuy croaker (*Miichthys miiuy*) (Shu et al 2016).

This study aimed to develop a model to study the interaction between PAMPs and PRRs of Nile tilapia using macrophages primary cell culture technique in order to further explore the molecular regulation of the immune response and reduce dependence upon *in vivo* disease studies in the Nile tilapia.

2.2 Material and Methods

2.2.1 Optimization of Macrophage primary cell culture conditions

2.2.1.1 Experimental animals

Nile tilapia (*Oreochromis niloticus*) were bred and reared in tropical aquarium (Institute of Aquaculture, University of Stirling). Fish were cultured in 1x1 m^2 tank in a recirculation system. The temperature of water was maintained at 28 °C and the light cycle was 12 hours light/12 hours dark. The fish were fed daily with a commercial diet and observed twice a day. The experimental fish were sacrificed following the Schedule 1 method which is an appropriate method for humane killing. Briefly, the fish were transferred to a bucket containing an anaesthetic, benzocaine, and the brain destroyed. All experiments were approved by the Animal Welfare and Ethical Review Body (AWERB) of the Institute of Aquaculture, University of Stirling, UK. Besides, each experiment was performed in 3 biological and technical replications

2.2.1.2 macrophages cell source and culture

In this study, head kidney, spleen and blood of Nile tilapia were collected for macropahges cell isolation and culture. The initial method of macrophage primary cell culture was a modification of a previous study in trout macrophages (MacKenzie et al 2003). Tissue samples and cell preparation; head kidney and spleen weighing approximately 15 mg were sampled using sterile forceps and scalpel, then kept in 50 mL tube containing 10 mL of Dulbecco's Modified Eagle's Medium-high glucose (DMEM) (Sigma-Aldrich, Dorset, UK) with 10% Chicken serum (CKS) (Gibco-BRL, Paisley, UK) and 100 µg/mL of Primocin (InvivoGen, Toulouse France). The tissue and 10 mL of culture medium were pressed through an 80

µm nylon mesh cell strainer to disassociate the tissue. Then cells were separated from the culture medium by centrifugation at 400 x *g* for 10 min. Supernatant was discarded and then, 10 mL of fresh culture medium, which contained DMEM medium, 10% of CKS and 100 µg/mL of Primocin, was added to re-suspend the cells.

Blood samples (2 mL) were extracted from the caudal vein with a heparin-coated syringe and needle. Monocytes/macrophages-like cells were obtained by density gradient centrifugation. Blood was diluted 1:1 with Phosphate Buffered Saline (PBS) pH 7.4 (Gibco-BRL, Paisley, UK) before being slowly layered into a 15 mL tube containing 3 mL of Histopaque® -1077 (1.077 g/mL) (Sigma-Aldrich, Dorset, UK) and centrifuged at 400 x *g* for 20 min. The interphase was collected and washed with 10 mL of PBS by centrifugation at 400 x *g* for 10 min. The cell pellet was re-suspended with 10 mL of culture medium, which contained DMEM medium, 10% of CKS and 100 µg/mL of Primocin. The cell suspension was seeded in triplicate wells into a the well plate (Nunc) (ThermoFisher, Scientific, UK) up to 2 mL which contained approximately 2-4x10^6 cell/well (1-2x10^6 cell/mL) and incubated at 28 °C with 3% CO_2.

2.2.1.3 Plate preparation

Poly-l-lysine (PLL) was used to coat the cell culture plate to enhance the adhesion between cell and culture plate surface. Plates were treated by adding 1 mL of 0.1% PLL (Sigma-Aldrich, Dorset, UK) into each well and rolling the plate until the surface was covered evenly with the reagent. The plate was incubated at 28 °C for 1 h and then all the reagent was removed by pipetting. After that, the plate was washed twice with 2 mL of PBS and left to dry. PLL treated plates were stored at 28 °C until use.

2.2.1.4 Cell and supernatant collection

In order to harvest the cells, scraping and pipetting were used. The cell suspension was transferred to an Eppendorf tube and then centrifuged at 400 x *g* for 5 min to separate the cells from the supernatant. 1 mL of supernatant was collected to a new tube and the excess amount of supernatant was discarded. 1 mL of TRI Reagent (Sigma-Aldrich, Dorset, UK) was added onto the cell pellet and mixed well by vortexing. The collection of supernatant and cell homogenate was stored in -20 °C for use

2.2.1.5 Cell stimulation

In this study, the macrophage primary cell culture was stimulated with ultra-pure Peptidoglycan (PGN) (InvivoGen, Toulouse France) to examine the effect of this bacterial PAMP on the cells. Macrophage cells, obtained from head kidney of Nile tilapia (n=3) were prepared and cultured in triplicate wells as described in section 2.2.1.2 for 3 days. Cells were stimulated by adding 10 µg/mL PGN into the cell culture media and gently stirring the plate for 10 s. Cells were incubated under culture conditions at 28 °C with 3% CO_2 for 6 h and 12 h before harvesting the cells and the supernatants. Cells were preserved in TRI Reagent (Sigma-Aldrich, Dorset, UK) for RNA extraction, while 1 mL of supernatant was transferred to an Eppendorf tube for the Prostaglandin E_2 Assay. Both homogenates and supernatants were stored at -20 °C.

2.2.2 RNA extraction

Samples preserved with TRI Reagent and stored at -20 °C were left to defrost at the room temperature and 100 µl of 1-bromo-3-chloropropane was added. The mixture was vortexed until the colour turned milky and samples were incubated for 15 min at room temperature. For phrase separation, the mixture was centrifuged at 13,000 x *g* for 15 min at 4 °C. 400 µl of the aqueous phrase was transferred to a new tube and 200 µl of isopropanol and 200 µl of RNA precipitation solution were added. The solution was mixed by inversion for 6-8 times and incubated at the room temperature for 1 h or -20 °C overnight. Samples were then centrifuged at 13,000 x *g* for 15 min at 4 °C. RNA pellets were washed with 1 ml of 75% ethanol. Samples were incubated at room temperature for 5 min before centrifugation at 13,000 x *g* for 5 min, ethanol was then discarded and pellets were dried by air-drying. Finally, an appropriate volume of DNAse/RNase free water was added for dissolution and samples were stored at -70 °C. RNA quality was measured with Nanodrop® ND-1000 (ThermoScientific, MA, USA). The RNA was then converted to a cDNA using the Precision nanoScript2 Reverse transcription kit (Primer Design, Chandlers Ford, UK). The cDNA working solution was diluted 7.5 times with RNAase/DNAse free water and stored at -20 °C. Finally, the quality of cDNA was estimated by PCR with β-actin primers.

2.2.3 cDNA synthesis

RNA was converted to a cDNA using the Precision nanoScript2 Reverse transcription kit (Primer Design, Chandlers Ford, UK). Based on the manufacturer's instructions, 1500 ng of RNA was used as a template for cDNA synthesis and the reaction was performed in a 0.2 ml tube. There were 2 steps in the reaction, in the first step; 1500 ng of RNA and 1 µl of Oligo-dT primers were added into 0.2 ml tube, then a final volume was made up to 10 µl with RNAase/DNAse free water. The mixture was heated at 65 °C for 5 min to allow the primers to anneal with the RNA template and then the tube was immediately transferred to ice. In the second step; a mixture was prepared with 5 µl of nanoscript2 buffer, 1 µl of 10 mM dNTP, nanoscript2 enzyme(reverse transcriptase) and 3 µl of RNAase/DNAse free water, the final volume was 10 µl. Then, 10 µl of the mixture was added to the tube on ice, mixed by pipetting and incubated at 42 °C for 20 min to synthesize the cDNA. After that the reaction was inactivated by heat at 75 °C for 20 min. The cDNA working solution was diluted 7.5 times with RNAase/DNAse free water and stored at -20 °C. Finally, the quality of cDNA was estimated by PCR with β-actin primers.

2.2.4 Primer design and determination of gene expression by PCR

For primer design, the sequence of the innate immunity-related genes of Nile tilapia; TNF-α, IL-1β, IL-6, IL-10, TGF-β and β-actin were downloaded from NCBI database (https://www.ncbi.nlm.nih.gov/). Specific primers were designed with Primer-blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) while, β-actin, a housekeeping gene, was used as standard primers to estimated the cDNA quanlity.

A PCR (Polymerase Chain Reaction) was conducted with an automated thermal cycler (Biometra®, Jena, Germany). The reaction of PCR consisted of 2 µl of cDNA (template), 1 µl of 10 mM forwards primer, 1 µl of 10 mM reverse primer, 10 µl of MyTaq™ Mix (Bioline, London, UK) and 6 µl of DNAse/RNAse free water, the final volume was 20 µl per reaction. According to a PCR protocol, the cDNA was pre-denatured at 95 °C for 1 min before synthesis. The PCR was set for 35 cycles with 3 steps; denaturation step at 95 °C for 15 s, annealiation step (the temperature depends on the annealing temperature of each primer pairs) for 10 s and extension step at 72 °C for 10 s. Then, final extension at 72 °C for 1 min to synthesize an incomplete amplicon. The PCR product was analysed by agarose gel electrophoresis. A volume of 1.5% w/v agarose gel (Biogene, Berkshire, UK) was

prepared with 0.5X Tris-acetate-EDTA (TAE) buffer and ethidium bromide. Then, 3 µl of PCR product was loaded into each well of agarose gel paralleled with a DNA marker (TrackIt™ 100 bp DNA ladder, Invitrogen™, CA, USA). The agarose gel electrophoresis was run in 0.5X Tris-acetate-EDTA (TAE) buffer at 70 V for 40 min and visualized the gel under UV light.

2.2.5 Prostaglandin E_2 (PGE_2) assay

Macrophage supernatants collected as described in section 2.2.1 were used for measurement of PGE_2 levels using a PGE_2 ELISA Kit-Monoclonal (Cayman Chemical, Michigan, USA). Macrophage supernatants were diluted with DMEM in a 1:1 dilution and prepared according to manufacturer's instructions. The assay is based upon competition between free PGE_2 and PGE_2 conjugated with acetylcholinesterase (AChE) (PGE_2 tracer) for a limited amount of monoclonal antibody. Briefly, an ELISA plate, coated with goat polyclonal anti-mouse IgG, was incubated with PGE_2 tracer, PGE_2 supernatant and PGE_2 monoclonal antibody at 4 °C for 18 h. The plate was developed with Ellman's reagent in the dark at room temperature for 60-90 min or until the supernatant became yellow using an orbital shaker. The assay was measured with a plate reader (Bioteck, Synergy HT, USA) at the wavelength between 405 and 420 nm. The concentration of PGE_2 was analyzed using the equation obtained from standard curve plot provided by the manufacturer.

2.2.6 Statistical analysis

PGE_2 concentrations between PGN stimulated and negative control cells were analysed using a paired t-test with a level of significance; $P<0.05$ and generated the graph using GraphPad Prism version 6.0 (San Diego, CA, USA).

2.2.7 Cell differentiation and phagocytosis assay

Flow cytometry was used to measure cell differentiation and the phagocytic activity of macrophage primary cell cultures. In brief, macrophages-derived from head kidney of Nile tilapia (n=3) were cultured in DMEM with 10% CKS and 100 µg/mL of Primocin using 6 well plate and incubated at 28 °C with 3% CO_2 for 4 days. Cell suspension of each fish was seeded in triplicate wells. In order to investigate the differentiation of monocytes to macrophages in primary cell culture, cells were harvested every day for 4 days. Experiments were performed in triplicate with 100 µL of cell suspension/replication. The flow cytometer (CytoFLEX, Backman Couther, USA) running condition was 60 µL/min and recorded all events. Discrimination of cell types was conducted with forward scatter (FSC) for cell size and side scatter (SSC) for cell complexity in a dot plot using CytoExpert software (Backman Couther, USA). In terms of a phagocytic activity assay, day 3 cells were harvested and re-seeded 200 µL of cell suspension in a 48 well plate (triplicate). Cells were incubated with 2 different solutions; heat killed *E.coli K-12* ($1X10^6$ cell/reaction) (Invitrogen™, CA, USA) and Zymosan (*S. cerevisiae*) ($1x10^5$ cell/reaction) (Invitrogen™, CA, USA) labeled with Fluorescein Isothiocyanate (FITC) incubatedat 28 °C with 3 % CO_2 for 1 h. After that, the plate was placed on ice to stop the phagocytic activity and cell supernatants were measured with the flow cytometer for fluorescence. The running condition was 60 µL/min and recorded all events. The phagocytosis data was measured by the intensity of FITC signal which has an excitation/emission stage at ~494/518 nm. Dot plot and histograms between FSC-A and Fluorescein FITC-A were generated to analyze the intensity and percentage of macrophages emitting fluorescence using the CytoExpert software (Backman Couther, USA).

2.3 Results

2.2.1 Optimization of Macrophage primary cell culture conditions

2.2.1.1 Macrophage cell source

In this experiment, head kidney, spleen and blood of Nile tilapia were used for optimization of suitable tissues for macrophage primary cell culture. In each experiment, there were 2 groups of samples and each group consisted of 3 fish (n=3). Head kidney and spleen were collected from group A, the average weight of fish was 100.4 ± 10.45 g, whereas in group B head kidney and blood were sampled from fish with an average weight of 189.2 ± 11.93 g. The cell suspension from head kidney, spleen, blood, a mixture of head kidney and spleen, and a mixture of head kidney and blood were seeded in triplicates into 6 well plates which had been treated with PLL with a cell number of approximately $2x10^6$ cells/well. The experimental design is attached in appendix 1. Macrophage-like cells were cultured at 28 °C, the same temperature as optimal rearing conditions for Tilapia, with 3% of CO_2. The culture medium was changed after 2 days of culture by removing the used medium and adding fresh medium into each well. Cells were observed daily for 7 days.

Visual examination revealed that the cell population was morphologically homogenous and viability of cells between each condition at day 1 was similar. However, the morphology of the cells changed on day 2 and more obviously on day 3 and day 4. After 2 days in culture, macrophage-like cell differentiation could be observed and the heterogeneous morphology of cells exhibited. The most cells were rounded, oval or pseudopod morphology. A pseudopod morphology was found next to a group of cells which suggested connection and mobility of the cells (Fig 2.1i). Cells (macrophage-like cells) were aggregating in 4-5 cell/groups by day 2 and became large groups of cells on day 3 which consisted of over 20 cells/group (Fig 2.1ii). The majority of cells were connected together and formed a granuloma-like structure on day 4 (Fig 2.1iii). In terms of cell adhesion, cells were attached to the culture surface on the first couple of days but de-attached upon day 3 of culture. De-attachment of the cells was obvious by day 4, a granuloma morphology was dominant.

Moreover, after changing the culture medium on day 2, more than 70% of cells were removed due to poor adhesion to the culture surface. At day 5, cell mortality was observed and the majority of cells

died by day 7 of culture. Cell mortality can be distinguished under the inverted microscope by a change in morphology and transparency of the cell. A typical macrophage-like cell morphology was rounded, oval, pseudopod or branched (adhered cell) and showed a bright colour (refraction) under the microscope. Whereas, dead cells were atrophied and non-transparent (Fig 2.1i). An overall performance (cell differentiation, cell adhesion and viability) for the macrophage-like cells generated from different tissues found that, the level of cell performance from each culture condition was blood > head kidney > a mixture of head kidney and blood > spleen > a mixture of head kidney and spleen. However, the number of cells generated from blood was approximately $6x10^5$ cell/mL which was approximately 3 times lower than head kidney and not enough to perform further work. Therefore, head kidney was the selected tissue to be used for macrophage primary cell culture.

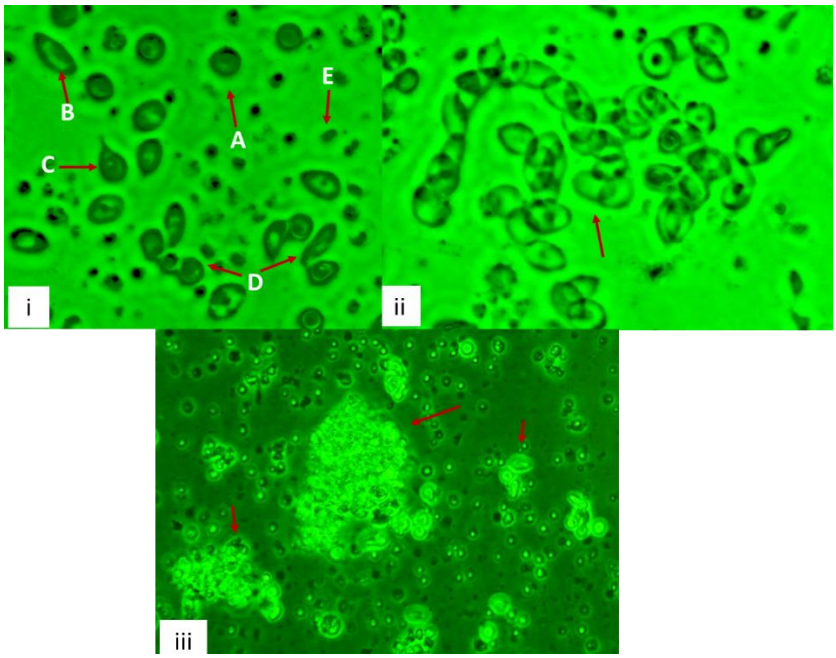


Figure 2.1 Macrophage primary cells derived from the head kidney Cells were plated in DMEM with 10% CKS and 100 µg/mL of Primocin, and incubated at 28 °C with 3% CO_2. Panel i: macrophage-like cell (day 2) showing cells with heterogeneous morphology. A: rounded cell, B: oval cell, C: pseudopod cell (moving cell), D: small group of cell, and E: cell debris or dead cell. Panel ii: a group of the macrophage-like cell on 3 days after seeding. Panel iii: macrophage-like cell formed granulomas after 4 days of culture. The image in panel i and ii were observed under X40 objective and panel iii was observed under X20 objective.

2.2.1.2 Effect of Poly-l-lysine (PLL) on macrophage-like cell differentiation

Poly-l-lysine (PLL) is an amino acid which is commonly used as a coating reagent in cell culture plates to increase binding sites for monolayer cell culture attachment. In a previous experiment (section 2.2.1.1; macrophage cell source), PLL had been used in order to increase the binding ability of the macrophage-like cells to the culture surface. However, only limited cell attachment was observed. Therefore, this experiment was designed to compare cell attachment between culture plates treated with PLL and without PLL. Head kidney of Nile tilapia was collected from 3 fish, an average weight of fish samples was 189.2 ± 11.92 g. Macrophage-like cells derived from the head kidney (~$2x10^6$ cell/well) were seeded in triplicate to 6 well plates which were treated with and without PLL (Appendix 1). Cells were incubated in the same conditions as in the previous experiment; 28 °C with 3% of CO_2 and observed daily for 7 days. The culture medium was changed on day 3 by removing half of medium in a well and adding an equal volume of fresh medium.

Results showed that there was limited cell attachment in both conditions. Cell adhesion was found in PLL treated plates after 2 days of culture (~20%) but not found in non- PLL treated plates. However, cells de-attached on day 3, exhibited a large group of cell which was formed from more than 20 cells/group, and granuloma-like structures were present on day 4. Whereas, the attachment of macrophage-like cells cultured in non-PLL treated plate occurred on day 3 and small groups of cells (4-5 cell) were observed at this time. However, cells in this condition de-attached on day 5 of culture and then formed cell chains and granulomas. Moreover, after we changed half of the culture medium on day 3, the number of cells decreased by approximately 50 % in both conditions, even though the cell cultured without PLL presented a cell adhesion on day 3 but there was only approx. 20% of the total cell population. Therefore, it was not possible to change the culture medium without reducing the number of cells. In terms of cell mortality, the mortality of cell cultured on PLL treated plates at Day 5 was ~40% higher than the cell cultured on non- PLL treated plates. The majority of cells died after 7 days of culture. Therefore, a non-PLL treated plate was chosen for macrophage primary cell culture in the further experiments.

2.2.1.3 Effect of different types and concentration of serum on macrophage primary cell culture

Serum provides essential factors which are necessary for growth and differentiation of the *in vitro* cell culture. In this experiment, different types of serum were used at a various concentrations to examine the effect of serum on the performance of the cells (cell differentiation, cell adhesion and viability). Two types of serum were tested in this experiment; Fetal Bovine Serum (FBS) (Gibco-BRL, Paisley, UK), which is widely used in cell culture, and Chicken Serum (CKS) (Gibco-BRL, Paisley, UK), which was used in the previous experiment. The concentration of each serum was 1 %, 5 % 10 % and 15 % respectively. Head kidney of Nile tilapia (n=3) was sampled, an average weight of fish samples was 167 ± 28.08 g. After cell preparation, the cell suspension was seeded in triplicate into 12 well plates without PLL coated (Appendix 1). Cells were incubated at 28 °C with 3% CO_2 and harvested on day6 of culture for examination of genes expression (TNF-α, IL-1β, IL-6, IL-10 and TGF-β) (Table 2.1).

Table 2.1 List of gene-specific primers with their product size, annealing temperature, and accession number of the gene

Genes	Sequence	Product Size	T_a	Accession number
TNF-α	>F CAGGATCTGGCGCTACTCAG	184 bp	60 ˚C	NM_001279533.1
	>R TAGCTGGTTGGTTTCCGTCC			
IL-1β	>F TGAGAGCCTACTTTAGGATTCTGC	150 bp	59 ˚C	XM_005457887.2
	>R GCGGCTATTACAACCAATGCT			
IL-6	>F CTGAGTGAGGGGAAAAGAGC	148 bp	61 ˚C	XM_019350387.2
	>R AGGAGTGTCAAAACCATCCAG			
IL-10	>F CTCAGATGGAGAGCAGAGGTC	134 bp	60 ˚C	KP645180.1
	>R CTTGATTTGGGTCAGCAGGT			
TGF-β	>F GAGATCCCTGCCAACTTGCT	230 bp	60 ˚C	NM_001311325.1
	>R TCCCCGACGTTACTCCGTAT			
β-actin	>F GCTACTCCTTCACCACCACAG	144 bp	61˚C	KJ126772.1
	>R CGTCAGGCAGCTCGTAACTC			

It was found that the cell cultured with FBS had a better performance than with CKS on day 1 and day 2. Cells in FBS showed 20 % more cell adhesion and cell connection than CKS. However, there was similarity in both serums on day 3 and day 4, as cell attachment in CKS increased forming more cell connections than day 2. After 5 days of culture, the mortality of cell in FBS was approximately 40%

higher than CKS (S. Ritchuay, personal observation). In terms of serum concentration, no differences were observed between dilutions on day 1 but differences were seen after day 2 in 1 % serum. On day 2, there was approximately 50% of cells dead but have no cells differentiation or cells connection in 1% serum condition.. On day 5, 10% serum was clearly the best concentration for the cell culture by demonstrating the largest number of living cells followed by 15%, 5% and 1%, respectively. The cells in 10% of CKS and FBS were harvested for RNA extraction and determination mRNA transcription level of selected genes by RT-PCR. Results showed that, mRNA transcription level of Tumor necrosis factor-α (TNF-α), Interleukin-10 (IL-10) and Transforming growth factor- β (TGF-β) were similar between the 2 culture conditions. However, a difference was found in Interleukin-1 β (IL-1β) and Interleukin-6 (IL-6). The mRNA expression of IL-1β and IL-6 analyzed was 2 times and 3 times higher in 10% FBS than 10% CKS (Fig 2.2). Across the cultures, pro-inflammatory gene expression was high (TNF-α, IL-1β, and IL-6) however anti-inflammatory expression was also very high (IL-10 and TGF-β) suggesting potential contamination during cell culture.

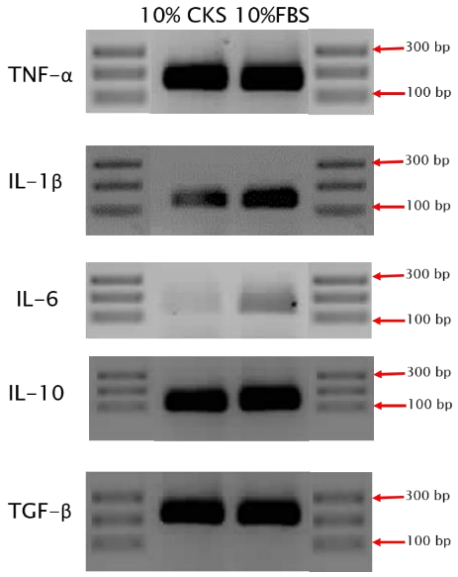


Figure 2.2 Gel electrophoresis image of macrophage-like cell cDNA amplified with innate immunity genes. Cells were cultured in 10% CKS and 10% FBS and incubated at 28 °C with 3% CO_2 for 6 days before harvesting. Lane 1: DNA ladder, Lane 2: PCR product of macrophage-like cell cultured with 10% CKS, Lane 3: PCR product of macrophage-like cell cultured with 10% CKS and Lane 4: DNA ladder

2.2.1.4 Effect of antibiotics and their concentration on macrophage primary cell culture

Contamination is a basic issue in cell culture and particularly for primary cell culture, as there are many risks of contamination during sampling and cell preparation. Therefore, to reduce the possibility of contamination from bacteria and Fungi, various types of antibiotics at different concentrations were tested to optimize the optimal conditions for macrophage primary cell culture. Head kidney was collected from 3 fish; average weight of fish samples was 240.3 ± 58.9 g and transferred to the culture medium without antibiotic. Macrophage cells derived from head kidney were seeded in a 12 well plate and cultured in culture medium containing different types of antibiotic. The experiment was performed in triplicates. Conditions tested were 100 µg/mL and 500 µg/mL of Primocin, 500 µg/mL of Kanamycin and 500 µg/mL of Penicillin/Streptomycin (Appendix 1). Cells were incubated at 28 °C with 3% CO_2 and harvested on day 3.

Our observations found that, there was no contamination during cell culture in all conditions. The types of antibiotic and their high concentration had no impact on the performance of the macrophage primary cell culture. There was a limitation of cell attachment and a group of cells (4-5 cells) were scattered over the plate on day 2. Cell chains and granulomas were found on day 3 and day 4. Cell mortality was found on day 5 and the majority of cell died after 7 day of culture in a similar manner to the previous experiments. After 3 days of culture, Cells were harvested to determine the level of mRNA transcribed from selected innate immunity genes by RT-PCR. Results showed that TNF-α, IL-10 and TGF-β were expressed in every condition while IL-1β and IL-6 showed the lowest level of PCR product particularly at 500 µg/mL of Primocin and 500 µg/mL of Penicillin/Streptomycin (Fig 2.3). Thus, 500 µg/mL of Primocin was chosen for cell culture in the further experiments. Interestingly, gene expression of studied genes was highly variable between individual fish. there were variation of genes expression between individual samples. Therefore, results suggested that the high levels of mRNA transcript were not due to contamination during cell culture but rather high regardless of the antibiotic used and highly variable at biological level.

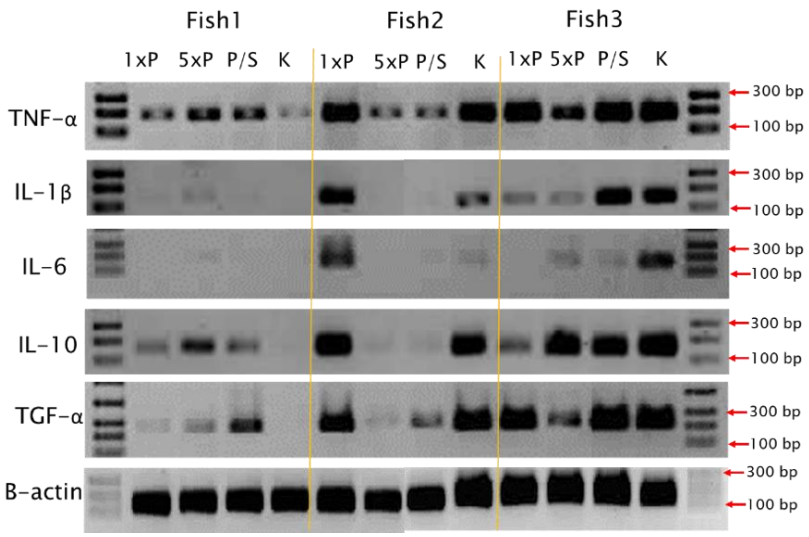


Figure 2.3 mRNA transcription level of selected innate immunity genes in macrophage-like cells cultured with different antibiotic conditions. Macrophage-like cells (n=3) were cultured in DMEM with 10 % CKS and various type of antibiotic, incubated at 28 °C with 3 % CO_2 for 3 days before harvesting. 1xP: 100 µg/mL of Primocin, 5xP: 500 µg/mL of Primocin, P/S: 500 µg/mL of Penicillin/Streptomycin, and K: 500 µg/mL of Kanamycin.

Observations made on cell cultures encompassing all experiments during the optimization process are summarized in Table 2.1. In each experiment, cells were cultured and their performance was observed for 7 days. Cells performance was evaluated by the level of cell viability, cell differentiation and cell adhesion. Performance was discriminated into 4 groups (good, normal, acceptable or poor). If more than 80% of cells were viable, the majority of cells were attached on a plate and a variety of cell morphologies were observed the cell culture was classified as having 'good performance'. In contrast, a 'poor performance' was defined as the condition where the majority of cells were dead and no cell attachment could be seend. 'Normal performance' was defined as the condition where ~70 % of the cells were alive, a variety of cell morphologies were observed and cells were attached on the plastic surface but still single cells could be seen floating in the media. Finally, performance was classified as 'acceptable' when around 50 % of the cells were alive and it was possibleto observe some of the cell adhesion and cell differentiation.

Table 2.2 A summary of macrophage primary cell culture performance which was cultured in different conditions. The performance of the cell was observed and recorded for 7 days. The performance of cell culture was assessed by a percentage of cell differentiation, cell adhesion and cell mortality. A level of cell performance was discriminated into 4 groups; Good (****), Normal (***), Acceptable (**) and Poor (*).

Factor	Experiments	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Tissue	Head kidney	*	***	**	**	**	*	*
	Blood	*	***	**	**	**	*	*
	Spleen	*	**	**	*	*	*	*
	Head kidney + Blood	*	**	**	*	*	*	*
	Head kidney + Spleen	*	**	**	*	*	*	*
Plate	PLL (+)	*	***	****	**	*	*	*
	PLL (-)	*	***	****	***	**	*	*
Serum	1% CKS	*	*	*	*	*	*	*
	5% CKS	*	**	**	*	*	*	*
	10% CKS	*	***	****	***	**	*	*
	15% CKS	*	**	**	*	*	*	*
	1% FBS	*	*	*	*	*	*	*
	5% FBS	*	**	**	*	*	*	*
	10% FBS	*	***	****	***	*	*	*
	15% FBS	*	**	**	*	*	*	*
Antibiotic	Primocin 100 µg/mL	*	***	****	***	**	*	*
	Primocin 500 µg/mL	*	***	****	***	**	*	*
	Kanamycin 500 µg/mL	*	***	****	***	**	*	*
	Penicillin/Streptomycin 500 µg/mL	*	***	****	***	**	*	*

2.2.1.5 Effect of ultra-pure Peptidoglycan on macrophages

According to the previous experiment (2.2.1.4), a high concentration of antibiotic was able to prevent a contamination during primary cell culture but some difficulty was found during RNA extraction process. Therefore, in this experiment we aimed to compare the RNA quality of the cells cultured in 2 different Primocin concentrations and to study the response of the macrophage-like cell to bacterial infection (PGN) which would facilitate a study in the next chapter.

Head kidney of Nile tilapia (n=3); with an average weight of 250.4 ± 46.07g, were collected and transferred to DMEM with 10% CKS and 500 µg/mL of Primocin and 500 µg/mL of Penicillin/Streptomycin to prevent a contamination during tissue sampling. For the primary cell culture, cells were separated into 2 groups; group A was cultured with DMEM containing 10 % of CKS and 500 µg/mL of Primocin and group B was cultured with DMEM containing 10 % of CKS and 100 µg/mL of Primocin. Both groups were seeded in triplicate into a 12 wells plate and were incubated at 28 °C with 3 % CO_2 for 3 days before stimulation with 10 µg/mL of ultra-pure PGN for 6 and 12 h, respectively (Appendix 1).

Before being activated with 10 µg/mL of ultra-pure PGN, cells were observed for 3 days to determine cell differentiation and viability. After 12 h of activation with PGN, visual examination of activated cell and negative control cells suggested that the activated cells presented higher cell mortality, while the negative control cells were normal as previously reported. The RNA quality of cell cultured with 500 µg/mL of Primocin was low, average RNA concentration was 50 ng/µL and 260/280 ratio was 1.7-1.8. However, it could be used for cDNA synthesis and PCR. An equal amount of RNA from each of the samples was used for cDNA synthesis and β-actin was used to evaluate the quality of cDNA. The result of PCR revealed unequal banding between samples (Fig. 2.4) which was not suitable to determine mRNA transcript of target genes.

In contrast, the average RNA concentration from the cell cultured with 100 µg/mL of Primocin was 300 ng/ µL and 260/280 ratio was 1.9-2.0. The amplification of the β-actin transcript presented equal bands in all samples (Fig. 2.5) which was suitable to perform RT-PCR. This suggested that using a high concentration of antibiotic may affect gene expression or RNA extraction method. Therefore, the final conditions of macrophage primary cell culture were DMEM with 10 % CKS and 100 µg/mL of Primocin, and incubate the cells at 28 °C with 3 % CO_2.

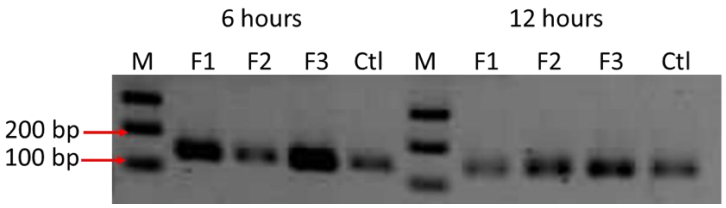


Figure 2.4 mRNA transcription level of β-actin in macrophage primary cells, cultured in DMEM with 500 µg/mL of Primocin. Cells on day 3 were activated with 10 µg/mL for 6 and 12 h. M: DNA ladder, F1, F2, F3; Fish1, Fish2, Fish3, respectively and Ctl: negative control.

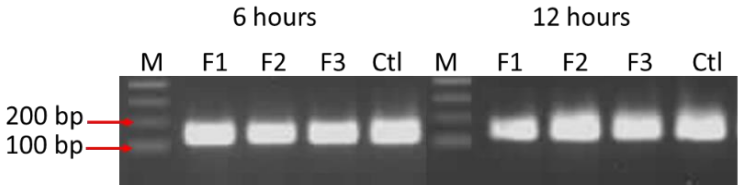


Figure 2.5 mRNA transcription level of β-actin in macrophage primary cells, cultured in DMEM with 100 µg/mL of Primocin. Cells on day 3 were activated with 10 µg/mL for 6 and 12 h. M: DNA ladder, F1, F2, F3; Fish1, Fish2, Fish3, respectively and Ctl: negative control.

2.2.2 Prostaglandin E_2 (PGE_2) assay

In order to determine the level of PGE_2, 1 mL of macrophage-like cell supernatant was collected after cell stimulation with 10 µg/mL of ultra-pure PGN for 12 h. The head kidney of Nile tilapia (n=6) was sampled for macrophage primary cell culture. Cell were cultured in DMEM with 10 % CKS and 100 µg/mL of Primocin and incubated at 28 °C with 3 % CO_2 for 3 days before stimulation. PGE_2 is produced by macrophages and released into the cell culture medium under activation (MacKenzie et al., 2010). A comparison of PGE_2 concentration between a stimulated cell and negative control cell showed that, there was no significant difference between the 2 conditions where *P*-value=0.9301 (α=0.05) (Fig.2.6). A mean of PGE_2 level obtained from stimulated cell was 77.72 ± 48.36 pg/mL (Maximum=137.83 pg/mL) and 77.33±49.34 pg/mL from non-stimulated cell (maximum=133.72 pg/mL).

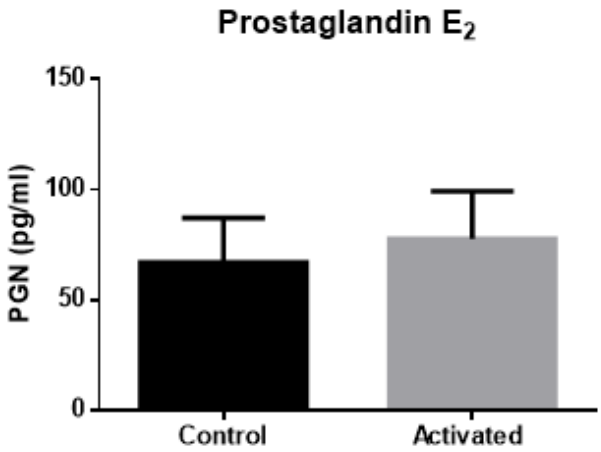


Figure 2.6 Level of PGE_2 upon stimulation with 10 µg/mL of PGN for 12 hours. Paired t-test was conducted to analyses the data with a level of significance; $P<0.05$.

2.2.3 Cell differentiation and phagocytosis assay

A phagocytosis assay was performed to investigate cell differentiation in the macrophage primary cell culture and to examine the phagocytic ability of the cultured cells. Phagocytosis is a major function of macrophages for the elimination of invading cells. The differentiation of macrophages-derived from head kidney was investigated during 4 days using flow cytometer analysis. It was found that during culture, the cells could be divided into 4 groups by their size and complexity which were lymphocytes, monocytes, differentiated monocytes and granulocytes (Fig. 2.7). On the first day of culture, there were 3 major groups of cell; lymphocytes, monocytes, and granulocytes (mean=17.98 %, 22.84 % and 34.85 % respectively), then the number of monocytes and granulocytes gradually reduced until almost disappearing on day 4 (mean= 4.54 % and 3.67 %). Whereas, a group of differentiated monocytes were growing every day from 8.46 % on day 1 to 38.53 % on day 4 (Fig. 2.7).

In terms of phagocytic activity, day 3 cells were incubated with *E.coli K-12* and Zymosan (*S. cerevisiae*) both conjugated with FITC for 1 h under control conditions (28 °C with 3% CO_2) before measuring the intensity of fluorescent emitting from macrophages. Results show that macrophages were able to engulf 23.35 % of *E-coli* and 20.05 % of yeast (Fig. 2.8).

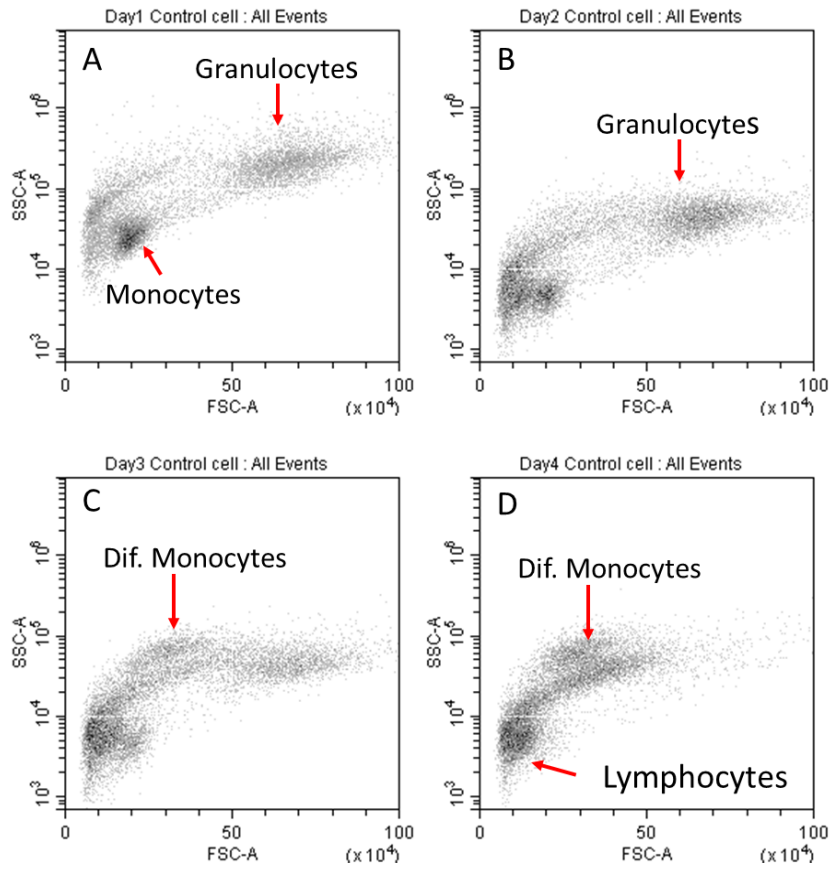


Figure 2.7 A dot plot between SSC-A and FSC-A of macrophage primary cell differentiation of Nile tilapia from the day 1-day 4 of culture. A: day 1, B: day 2, C: day 3, D: day 4

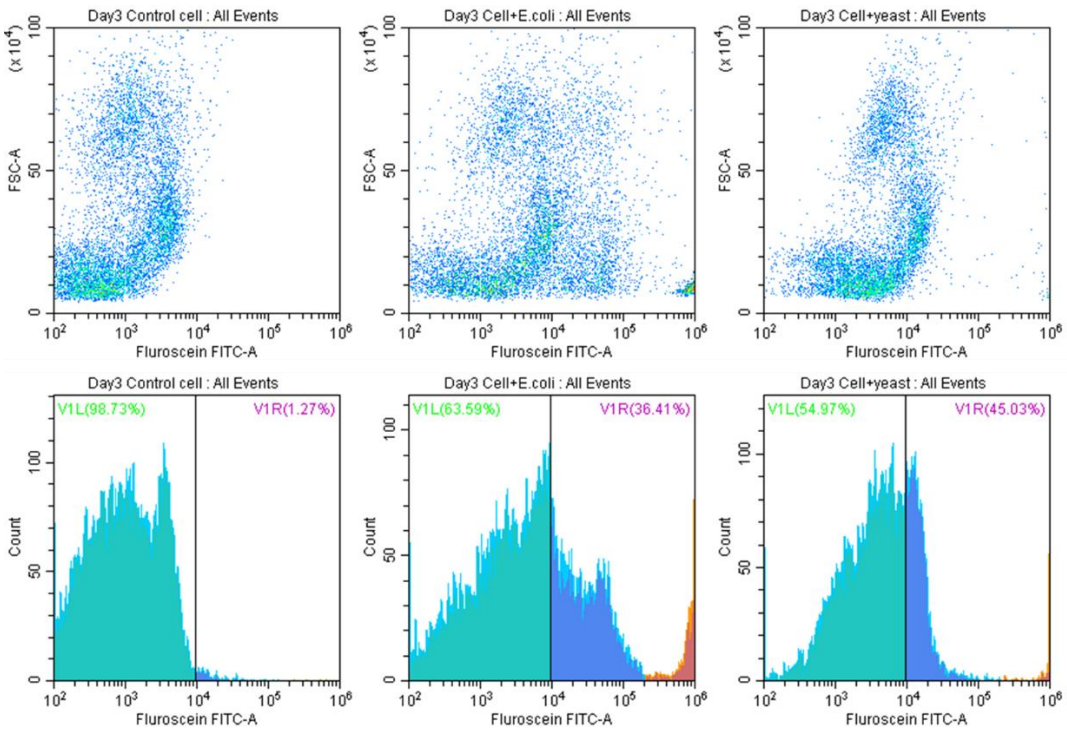


Figure 2.8 Dot plot between FSC-A and Fluorescein FITC-A and a histogram of macrophage primary cell culture, showing fluorescent intensity area and percentage of the phagocytic cell. Green: negative control cell, Blue: phagocytic cell, Orange: *E.coli* or Zymosan conjugated with FITC.

2.4 Discussion

2.4.1 Macrophage primary cell culture

Macrophages are a key orchestrator of the immune response as they have a phagocytic activity to detect and eliminate invading pathogens. Moreover, macrophages are a major source of cytokines that are the critical factor involved in the communication between the innate immune system and the adaptive immune system of the host. As macrophages are robust cells with many functions in immunity, they have been used as a model to determine the interaction between PAMP and PRR in many studied by using cell culture techniques such as rainbow trout (MacKenzie et al 2003, Hong et al 2003 and Costa et al 2011), European eel (Callol et al 2013), goldfish (Grayfer et al 2011 and Haddad et al 2008), grass carp (Wang et al 2016) and Miiuy croaker (Shu et al 2016). Primary cell culture is a

useful technique to study the function of cells which are obtained from fresh tissue (also known as ex-vivo culture). Cell culture conditions are optimized in order to enrich for a specific cell population from the target tissue. This can be done for a variety of cells which have no cell line available (Unchern 1999). Importantly, specific cellular functions are maintained in primary culture as opposed to cell lines that may lose functions during immortalisation. However, as the cells have a limited life span there is a need to isolate cells from fresh tissue for every new experiments (Unchern 1999).

In the current study, the condition factors for macrophage primary cell culture in Nile tilapia were optimized. The monocyte-macrophage like cell rich tissues in Nile tilapia (head kidney, spleen and blood) were collected to develop the culture. Culture conditions were developed based on a previous study in trout macrophages (MacKenzie et al 2003). Multiple factors of culture conditions which could affect cell survival and function were optimized in order to best mimic the *in vivo* environment of the cells. These were cell source, cell adhesion, and concentration and type of serum and antibiotic. The temperature, CO_2 and culture media conditions were DMEM (high glucose) with incubation at 28 °C (the same as rearing conditions) with ~3-5% CO_2. The performance of the primary cell culture (cell differentiation, cell adhesion and cell mortality variables) were visually observed under the inverted microscope for 7 days.

In terms of cell source, the monocyte/macrophage rich tissues (blood, head kidney and spleen) were cultured in both monoculture and co-culture. Our results show that cells cultured from a monoculture source (i.e. the same tissue) have a better performance than co-cultured cells. The head kidney, the main hematopoietic tissue, was the most suitable source for macrophage primary cell culture. The co-culture was used to examine whether an interaction between the cells from different tissues could improve culture performance. In general, the co-culture of the cell is conducted to study the interaction between the cell populations and to improve the culturing success of the main population as some types of cell are difficult to be cultured in single population or cannot exhibit their natural behaviour as in the *in vivo* environment (Goers et al 2014). The co-culture of macrophages have been conducted in many studies, mostly in human macrophages for an examination of inflammatory response and cell to cell interaction for medical purposes (Bodet et al 2006; Kamoshida et al 2012; Roudnicky and Hollmén 2016).

However, in this experiment, the cells cultured under co-culture, mixed tissue, conditions (head kidney + blood and head kidney + spleen) did not improve the cultivation success but induced significant cell mortality on day 4 of culture. In contrast, the mortality of cell from single tissue sources extended

beyond 4 days of culture. Our results suggest that macrophages derived from blood had the best performance when compared to the cells derived from head kidney and spleen, but the number of cells obtained was less than those derived from the head kidney (Table 2.1). The number of monocyte/macrophage cells depend on the tissue where they are derived. In teleost, the head kidney is the main hematopoietic tissue which produces erythrocytes, thrombocytes, lymphocytes, monocytes and granulocytes. The monocytes are released into the bloodstream and then develop into macrophages after they migrated into tissue (trans-endothelial migration). Thus, blood is a suitable tissue for monocyte collection while macrophages can be collected from spleen however the low number of cells derived is a serious limitation when compared to head kidney which produces a large number of monocytes and their precursors. Thus, it could be implied that the cells derived from different tissues have different cell behaviour which is a consequence of the observations of the individual cell cultures. In line with this, we observed that the cells derived from blood presented cell mobility and cell connection on day 2 whereas those cells could be observed on day 3 in the cell-derived from the head kidney. This is likely related to the different cell differentiation status.

Cell adhesion was optimized by comparing cells cultured in PLL treated plates and non-PLL treated plates. We found that PLL treated plates did not increase the cell attachment but did induce cell differentiation and mortality which was approx. 40% higher than non PLL-treated plate. Poly-l-lysine (PLL) is an amino acid which is commonly used as a coating reagent on cell culture plates to increase binding sites for monolayer cell culture attachment. It does this by increasing electrostatic interactions between negatively-charged ions of the cell membrane and positively-charged ions of the culture surface. However, pH and temperature affect the formation of PLL structures which change the stability of the polypeptide chain (Mirtič and Grdadolnik 2013), thus might interrupt cell binding ability. Furthermore, the behaviour of cells obtained from different tissues tends to have different adhesion behaviours. The cells derived from blood, spleen or bone marrow adhere poorly in the culture plate as they are in suspension or loosely adherent in the body (Unchern 1999). However, it was found that monocytes roll along the vascular surface or adhere to the epithelium before migration from bloodstream into tissue (Maslin et al 2005) thus this process may be promoted by PLL. Different surface coating e.g. PLL, fibronectin and collagen have been shown to stimulate monocyte-macrophage adhesion thus enhancing cell capture in primary culture systems. A recent study reported that fibronectin induces human macrophages migration in a dose-dependent manner (Digiacomo et al 2017). However, further studies into temperature-dependent adhesion onto specific-coating on the culture plate for fish are required.

In terms of culture media, 2 types of serum (CKS and FBS) were compared in various concentrations. Results revealed that FBS induced a higher level of IL-1β and IL-6 mRNA abundance when compared to CKS suggesting that FBS is a more potent cell stimulant than CKS or it may has been contaminated. Interestingly, a 10% concentration of serum showed the best cell performance when compared to 1%, 5% or 15%, causing massive cell mortality. Therefore, 10% CKS was a suitable serum for macrophage primary cell culture of Nile tilapia. Serum is a supplement to the basal growth medium in cell culture; containing a large range of essential nutrients for cell growth. It is derived from whole blood but red blood cells and other clotting components are removed. The concentration used depends on a particular cell line, and batches of serum have different qualities (Unchern 1999). It has been reported that Human serum and FBS influence different behaviour of human cervical cancer cell lines SiHa and HeLa (Heger et al 2018) suggesting the use of different serum can introduce a variety of cell behaviour. As there is a significant evolutionary difference between mammals, birds and fish it is likely that both mammalian and avian serums do not contain an optimal set of growth promoting molecules for fish. Following this line of thought, trout serum was used to enhance culture in a zebrafish cell line (Ruyra et al 2015). However, in some case, a fish serum cannot improve culture but induce cell mortality when using its own serum (Pers.Comn.,2018).

Finally, antibiotic concentrations were optimized for Primocin, Kanamycin and Penicillin/Streptomycin (100 µg/mL and 500µg/mL). It was found that 100 mg/mL of Primocin was the best concentration for primary cell culture as a higher concentration of this antibiotic (500µg/mL) had a negative effect on the RNA extraction process, RNA yield and quality and hindered gene expression studies. Antibiotics are necessary to prevent bacterial and fungi contamination in cell culture media. They are generally active against bacteria, mycoplasma or fungi by blocking DNA and protein synthesis or breaking cell membranes. It is known that antibiotics, under ideal conditions, should selectively eliminate bacteria but not host cells due to a specific activity and a proper usage dose, which is not harmful to the host cell (Mobley 2006). However, during culture, molecular regulation of cell function may change because of a non-optimal environment that influences cell sensitivity and susceptibility. Some antibiotics such as cycloheximide (CHX) and lactimidomycin (LTM), which have antifungal and antiviral properties, have a strong effect and inhibit the translation process of eukaryotic cells therefore high concentrations affect translation and protein production (Schneider-Poetsch et al 2010). Therefore, an overdose of antibiotic might not useful but in fact toxic to the cell culture.

Primary cell cultures have a high risk of bacterial and fungal contamination as cells are isolated from fresh tissues. In order to minimize the risk of contamination, an appropriate concentration of antibiotic during culture is required. Therefore, during tissue sampling, a concentrated dose of antibiotic was used; a mixture of 500 µg/mL of Primocin and Penicillin/Streptomycin to reduce the risk of contamination. The working concentration for the cell culture was then reduced to 100 µg/mL of Primocin to maintain sterile conditions and reduce toxicity. Primocin was the most effective antibiotic for tilapia macrophage primary cell culture providing a complete defense against bacteria mycoplasma and fungi (InvivoGen, Toulouse France). On the other hand, Penicillin/Streptomycin which is a common antibiotic widely used in cell culture, was not effective in our culture system. 100 µg/mL of Penicillin/Streptomycin has been reported to cause mortality in melanoma cells whereas using kanamycin or gentamicin had less effect (Martínez-Liarte et al 1995). The use of antibiotics in cell culture is necessary and widespread however in the case of primary culture our data shows that it is essential to optimize antibiotic concentrations in order to avoid unwanted side-effects.

2.4.2 Functions of macrophages

According to the development of macrophage primary cell culture of Nile tilapia described here, the characteristics and function of the macrophage-like cells obtained were observed by analyzing PGE_2 production and phagocytic activity. PGE_2 secretion was measured after cell stimulation with PGN for 12 hours compared to that of non-stimulated cells. PGE_2, is a lipid mediator that plays a crucial role in the inflammatory response. It is produced from macrophages through the COX2 pathway after macrophage activation via PAMPs, hormones and growth factors (Ricciotti and FitzGerald 2011). In general, the accumulation of prostaglandin is at a very low level in healthy tissue but increases immediately after an infection event and tissue/cellular damage (Ricciotti and FitzGerald 2011).

In human peripheral blood monocytes, PGE_2 was detected in both control and LPS activated conditions, and the small amount of PGE_2 found in control cells was drastically increased after LPS activation (Niho et al 1998). A similar result was found in human polymorphonuclear leukocytes (PMN) after induced with PGN (Valera et al 2007) and monoblastic leukemia cells (U937 strain) show a PGN dose-dependent secretion of PGE_2 (Tanabe and Grenier 2008). In parallel, a study in rainbow trout macrophages reported that the level of PGE_2 was very low when absent of PAMP, but was significantly increased

after activation, approximately 10 times. Production of PGE_2 stimulated with PGN was slightly delayed when compared to LPS stimulation (MacKenzie et al 2010). Similar studies in mammalian systems, for example, RAW 264.7 mouse macrophages, display a concentration and time-dependent induction of COX-2 and PGE_2 production when stimulated with PGN with IL-6 production being involved in COX2 generated PGE_2 (Chen et al 2006). The above examples and the significant body of available literature strongly suggests that the PAMP-driven PGE_2 secretion and regulation is highly conserved across the vertebrates.

In this study contrasting results were found, as there was no measurable difference between PGE_2 production after PGN stimulation and controls. The level of PGE_2 in the supernatant of PGN-stimulated and non-stimulated cell were measured, an average amount was 77.72 pg/mL and 77.33 pg/mL, with the highest amount analyzed at 133.72 and 137.83 pg/mL respectively. In terms of acute inflammation, the amount of PGE_2 secreted from non-stimulated tilapia macrophages was relatively high when compared to basal production in humans (Niho et al 1998) or rainbow trout (MacKenzie et al 2010). The result indicates an activated state of the cultured macrophages, which might be the impact of growth factors in culture media or a contamination issue. Moreover, in this study, PGN stimulation could not induce PGE_2 production. This suggests that expression of COX-2 might be suppressed by cytokines during chronic infection to promote the healing/resolution stage of inflammation. As PGE_2 and inflammatory cytokines are active in an autocrine manner their cross-regulatory roles have been reported in many studies, particularly in human models. For example, a study by Niho et al (1998), reported IL-10-driven suppression of PGE_2 production in human monocytes after stimulation with LPS. On the other hand, as PGE_2 involves both immune activation and suppression, a PGN-induced concentration and time-dependent effect of IL-6 caused PGE_2 production in mouse macrophage cell line, RAW 264.7 (Chen et al 2006). Moreover, it has been reported that a subclinical infection or chronic inhibition of COX-2 in macrophages induced inflammation in the host due to a reduction of PGE_2. This may cause chronic inflammation during the healing stage and is a mechanism exploited by intracellular parasites; *Mycobacterium tuberculosis* can induce PGE_2 secretion in macrophages to suppress inflammation and support their survival (reviewed by Hamidzadeh et al 2017). Thus, there are several factors involved in macrophage PGE_2 production and requires further study to discover the hidden cause of the high basal levels of PGE_2 production in our macrophage model and its lack of response to bacterial PAMPs.

In terms of cell differentiation and phagocytic ability, flow cytometry was used to observe cell differentiation for 4 days post-seeding and the phagocytic ability of the cells examined against *E.coli K-12* and Zymosan (*S. cerevisiae*) at 3 days of culture. It was found that the macrophage primary cell culture derived from head kidney comprised of at least 3 populations; monocytes, granulocytes and lymphocytes. The number of monocytes and granulocytes gradually decreased during the cultured period, whereas a new population of cells increased. Monocyte to macrophage differentiation occurred as there was an increase in the macrophage population size while the monocyte population decreased. Moreover, the loss of the granulocyte population during the culture period has been previously been reported and is due to the short life span of the granulocytes and neutrophils which survive less than 1 day in the bloodstream (McCracken and Allen 2014). On the other hand although not observed in this study human eosinophils can exist for approximately 18 hours and survive at least 6 days tissue (Park and Bochner 2010). Sub-populations of cells observed during the macrophage differentiation of Nile tilapia was relatively similar to that reported in carp (*Cyprinus carpio)* (Joerink et al 2006), European eel (*Anguilla Anguilla*) (Callol et al 2013), and Lumpsucker (*Cyclopterus lumpus*) (Haugland et al 2012). However, a dissimilarity between this culture and others is the culture temperature (28°C), where higher temperatures induce faster cell growth and cell mortality when compared to cold-water fish species.

The primary function of macrophages is the pathogen elimination by phagocytosis and contribute the immune response by humoral signal (Callol et al 2013). The capacity of engulfment is supported by a wide range of receptors that recognized a variety of foreign and endogenous ligands (Lim et al 2017). In this study, the phagocytic ability of cultured cell was investigated on day 3 of culture to examine the capability of macrophages to phagocytose either *E.coli K-12* or Zymosan (*S. cerevisiae*) which represent extracellular and intracellular parasites. 3 days of culture was the minimum period for macrophage differentiation and the majority of other phagocytic cell such as neutrophils and eosinophils are not present at this point therefore providing an analytical window to study macrophage phagocytosis at this time. Results showed that macrophages derived from head kidney were able to phagocytose both of *E.coli K-12* and Zymosan (*S. cerevisiae*) efficiently. The percentage of *E.coli K-12* engulfed by the macrophage was slightly higher than that of Zymosan (23.35% and 20.05%, respectively). The percentage of phagocytosed bacteria of this study was high when compared to European eel (Callol et al 2013) and carp (Joerink et al 2006), but the efficiency of yeast phagocytosis was low when compared to the murine macrophage-like cell line J774.1 (Carneiro et al 2014) or European eel (Callol et al 2013). This discrepancy might be an effect of contamination as a pre-stimulation with LPS increased bacterial

phagocytic ability of carp macrophages from 13% to 23% (Joerink et al 2006). Moreover, the capability of pathogen uptake of the macrophages depends upon cytokine stimulation and the cytokines present in the culture supernatant will impact upon phagocytic activity. For example, the phagocytic ability of rainbow trout macrophages was induced by rIL-1β (Hong et al 2003) and rTNF-α in a dose-dependent manner (Zou et al 2003). Although cytokine-driven regulation of phagocytic activity in fish macrophages is important the interaction between these molecules and cellular activity have been poorly studied in fish and require further study.

Importantly, the formation of granulomas observed in the tilapia macrophage cell culture is another interesting point in this study. This phenomenon has not been widely reported in cell culture literature. The granuloma is an aggregation of macrophages and other immune cells during the inflammatory response to control and contain foreign particles such as intracellular parasites. Certain bacteria can induce granuloma formation in the host by disturbing macrophage functions including; cytokine and PGE production and phagocytic activity to generate a suitable environment for their survival (reviewed by Grayfer et al 2011). Therefore, we suggest that another possible reason for granuloma formation in the Tilapia macrophage cell culture, high basal secretion of PGE_2 and phagocytic activity might cause by intracellular parasite infection. This will be explored in later chapters.

2.5 Conclusions

In this study we have developed a macrophage primary cell culture derived from head kidney tissue of NIile tilapia. Optimal conditions for primary cell culture were DMEM high glucose with 10% CKS and 100 µg/mL of Primocin and cells incubated at 28 °C with 3 % CO_2. Whereas, the media for sampling required a high amount of antibiotic to sterilize the tissue; DMEM high glucose with 10 % CKS, 500 µg/mL of Primocin and 500 µg/mL of Penicillin/Streptomycin. The functions of macrophages produced in the culture; PGE_2 production and phagocytosis were observed and cells successfully phagocytosed *E.coli K-12* and Zymosan (*S. cerevisiae*) which is a major function of macrophages. However, the amount of PGE_2 in non-stimulated cells and no observable stimulation with PGN suggested that PGE_2 production might be activated by growth factors correlated to an unstable attachment leading to granuloma formation during culture. This also might be an effect of pathogen contamination, which needs to be evaluation.

Chapter 3

Characterisation of PAMP-PRR interactions in Nile tilapia (*Oreochromis niloticus*) using a macrophage primary cell culture

3.1 Introduction

The innate immune system is an important defence system of fish against pathogens infection as they live in a high pathogenic pressure environment. The innate immune responses are the first line of defense which rapid react and relies on a highly conserved component's structure on surface or inside the pathogen referred as Pathogen Associated Molecular Patterns (PAMPs) such as lipopolysaccharides (LPSs) found in Gram-negative bacteria, peptidoglycans (PGNs) found in both Gram-positive and Gram-negative bacteria, -glucans of fungi, and viral nucleic acids (MacKenzie et al 2010). PAMPs are recognized by specific Pattern Recognition Receptors (PRRs) present on a range of host immunity cells such as macrophages, dendritic cells, neutrophils and natural killer (NK) cells. In fish, 4 groups of PRRs have been described; Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLR), C-type lectin receptors (CLRs) and peptidoglycan recognition receptors (PGRPs) (Boltana et al 2011). These receptors are activated after pathogen infection and play as key role to initiate the inflammatory responses by the production of cytokines and chemokines which trigger an adaptive immune system of the host.

Cytokines are soluble proteins, mainly produced by macrophages and lymphocytes. According to their function, cytokines can be classified into 2 groups; pro-inflammatory cytokines and anti-inflammatory cytokines. The pro-inflammatory cytokines bind to a specific cell surface receptor to generate a cell signalling cascade and trigger Th1 to stimulate an adaptive immune response (Duque and Descoteaux 2014). Whereas, anti-inflammatory cytokines work as immunosuppressive molecules by inhibiting pro-inflammatory cytokines production which results in Th2 development and tissue regeneration. The

regulation of inflammation is necessary for host defense and homeostasis, but uncontrolled PRR or cytokines secretion can lead to severe tissue damage and chronic illness states (Srinivasan et al 2017). This means that the upregulation or downregulation of those molecules have profound effects on the immune response.Therefore, understanding the expression dynamics and interactions between PAMPs and PRRs are essential for health and disease management, particularly in Nile tilapia (*Oreochromis niloticus*), which is commercially important and it is considered a disease tolerant species. However, available knowledge related to the molecular and cellular immune response of this species is limited.

Primary culture of macrophages is a valuable tool to use as a model to study the effect of PAMPs on host immunity as the macrophages are the key orchestrators of the immune response. Macrophages have phagocytic activity in order to detect and eliminate invading pathogens and they are also essential for tissue development and homeostasis. Moreover, macrophages are a major source of cytokines that are the critical factors involved in the communication between innate immunity and adaptive immunity of the host. Macrophages are derived from hematopoietic stem cells like monocytes and circulate in the bloodstream. In fish, monocytes are generated in the head kidney. Monocytes penetrate through blood vessels into tissues and then differentiate to macrophages. In general, macrophages are stable at a resting state but they can be activated by a series of PAMPs. Activated macrophages (M1) secrete pro-inflammatory cytokines to promote inflammation and the macrophages themselves play as an antigen-presenting cell to trigger Th1 lymphocytes. While the alternatively activated macrophages (M2) produce anti-inflammatory cytokines which promote a healing state after infection and regulate the production of pro-inflammatory cytokines.

In teleost, macrophage primary cell cultures have been used to study the immune response in a number of species. For example, macrophages derived from kidney of goldfish (*Carassius auratus*) were used in order to characterize the interaction between *M. marinum* and phagocytes. This study revealed an upregulation of pro-imflammatory genes mRNA after exposure to *M. marinum*, however a survival rate of intracellular mycobacteria reduced after a stimulation of macropahges with recombinant goldfish (rg) TNF-α2 or rgINFγ (Grayfer et al 2011). This model was also useful to characterize inflammatory cytokines function such as TNF-α and its role in inflammatory cytokines network in rainbow trout (*Oncorhynchus mykiss*) (Hong et al 2013). A similar study was done to study the role of TGF-β in goldfish (*Carassius auratus*) (Haddad et al 2008) and IRF3 in Miiuy croaker

(*Miichthys miiuy*) (Shu et al 2016). Besides, mechanisms invoved in the recognition by host PRRs and their inflammatory response upon different PAMPs was done in rainbow trout (*Oncorhynchus mykiss*) (MacKenzie et al 2006) and European eel (*Anguilla anguilla*) (Callol et al 2013) using macrophage primary cell culture. These studies reveal the potential to use macrophage primary cell cultures as a model to study the molecular and cellular immune responses in fish.

Therefore, in this chapter, a macrophage primary cell culture system which was developed on chapter 2 will be used as a model to characterize the interaction between PAMPs and PRRs in Nile tilapia. This study aims to provide a knowledge platform to understand the molecular regulation of the immune response and reduce dependence upon *in vivo* disease studies in Nile tilapia.

3.2 Material and Methods

3.2.1 Molecular tool development

3.2.1.1 Primer design

The mRNA sequences of innate immunity-related genes of Nile tilapia; TNF-α, IL-1β, IL-6, IL-10, TGF-β, Viperin, IRF3, TLR2, TLR3, TLR8 and TLR9 were downloaded in FASTA format from NCBI database (https://www.ncbi.nlm.nih.gov/) and specific primers design conducted with Primer-blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) which is an online software supported by NCBI (Table 3.4). Primer-blast program setting was; the melting temperature of primers was arranged from 55 °C to 63 °C and no more than 3 degree difference between the melting temperatures of 2 primers. The minimum and maximum size of product were 100 and 300 base pairs (bp), respectively. A Self-complementary of primer was investigated using Multiple primers analyzer which is an online available program (https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/ thermo-scientific-web-tools/multiple-primer-analyzer.html). Selected primers were then synthesized by Eurofin Genomics Company. The primers pellet were dissolved with DNAse/RNAse free water and diluted to 10 mM as a working solution for PCR.

3.2.1.2 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction was performed to optimize an annealing temperature of primers and to evaluate primer efficiency before being applied to examine the mRNA expression level of target genes. A PCR reaction consisted of 2 µl of cDNA (template), 1 µl of 10 mM forwards primer, 1 µl of 10 mM reverse primer, 10 µl of MyTaq™ Mix (Bioline, London, UK) and 6 µl of DNAse/RNAse free water, the final volume was 20 µl per reaction. Prior DNA synthesis, a PCR mixture was heated at 95 °C for 1 min to completely denature a double-stranded DNA. The PCR was synthesized for 35 cycles through 3 steps which were; denature step at 95 °C for 15 s, annealing step (the temperature depends on the annealing temperature of each primer pairs) for 10 s and extension step at 72 °C for 10 s. Then final extension at 72 °C for 1 min to synthesize an incomplete amplicon (Table 3.1). The PCR was conducted with an automated thermal cycler (Biometra®, Jena, Germany). The PCR product was analysed by agarose gel electrophoresis. A volume of 1.5% w/v agarose gel (Biogene, Berkshire, UK) was prepared with 0.5X Tris-acetate-EDTA (TAE) buffer and ethidium bromide. Then, 3 µl of PCR product was loaded into each well of agarose gel paralleled with and a DNA marker (TrackIt™ 100 bp DNA ladder, Invitrogen™, CA, USA). The agarose gel electrophoresis was run in 0.5X Tris-acetate-EDTA (TAE) buffer at 70 V for 40 min and visualized the gel under UV light. The PCR products with a single clear band were purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Duren, Germany) and cloned for preparing qPCR standard plasmid.

Table 3.1 Polymerase Chain Reaction (PCR) protocol

Component	1 reaction
Template	1 µl
10 mM forwards primer	1 µl
10 mM reverse primer	1 µl
PCR mix (mytaq)	10 µl
DNAse/RNAse free water	7 µl
Final volume	20 µl

Step	Temperature	Time	
Pre-denaturation	95 °C	1 min	
Denaturation	95 °C	15 sec	35 cycles
Annealing	56-61 °C	10 sec	
Extension	72 °C	10 sec	
Final extension	72 °C	1 min	

3.2.1.3 Cloning and standard plasmid preparation

A purified PCR product was ligated into pGEM®-T Easy Vectors (Promega, Southampton, UK) before the transformation. The transformation reaction was performed with a competent cell (DH5α). A volume of 2 µl of ligation reaction and 50 µl of competent cells were gently mixed and allowed to stand on ice for 20 m. Cells were heat-shocked at 42 °C for 45 se to uptake the vector and immediately placed on ice for 2 min before adding 250 µl of LB broth. Competent cells were incubated at 37 °C with approximately 150 rpm shaker for 2-3 h. For colony selection, cells were cultured on LB agar with 50 mg/ml of ampicillin at 37 °C overnight. Bacterial colonies were collected individually and grown in 500 µl of LB broth with 50 mg/ml of ampicillin, then incubated at 37 °C with approximately 150 rpm shaker for 3 h. A colony PCR was performed to confirm insertion of the target fragment. After that, the PCR positive colony was selected for plasmid extraction using NucleoSpin Plasmid EasyPure (Macherey-Nagel, Duren, Germany). The plasmid quality was measured with a Nanodrop® ND-1000 (ThermoScientific, MA, USA) and stored at -20 °C. The target fragment was ensured by sequencing with T7 promoter primer (5'-TAATACGACTCACTATAGGG-3'). The copy number of plasmid was calculated based on the followed formula:

$$Number\ of\ copies/\mu l = \frac{M \times 6.02 \times 10^{23} \times 10^{-9}}{N \times 660}$$

Where (M) is an amount of plasmid concentration in nanogram (ng), (N) is a number of nucleotides in plasmid included with pGEM-T vector (3015 bp) and insertion fragment size and 660 Da is the nucleotide weight. By following the formula, a copy number of standard plasmids was calculated and a series dilution generated (10^8-10^2) for qPCR standard and stored at -20 °C until use.

3.2.2 Evolutionary conservation

mRNA sequences of target genes (TNF-α, IL-1β, IL-6, IL-10, TGF-β, Viperin, IRF3, TLR2, TLR3, TLR8 and TLR9) from Nile tilapia and other different organisms were obtained in FASTA format from the NCBI database (https://www.ncbi.nlm.nih.gov/). An accession number of the sequences is provided in

appendix 2. Sequences were aligned and calculated a distance between each organism based on the difference of sequences between taxa to find the similarity among those taxa by UPGMA (Unweighted Pair Group Method using arithmetic Average). Finally, the phylogenetic trees were constructed with a Bootstrap to determine the reproducibility of the tree. A sequence of β-actin of Nile tilapia was used as an out group. The UPGMA calculation and Phylogenetic tree construction were performed with MEGA X software (Institute of Molecular Evolutionary Genetics, The Pennsylvania State University, USA).

3.2.3 PAMP-PRR interactions

3.2.3.1 Macrophage cell stimulation

In this study, the macrophage primary cell culture of Nile tilapia was stimulated with 2 stimulation reagents (PAMPs); PGN and poly I:C, to examine the response of target genes. In the first experiment, macrophage-like cells were isolated from head kidney of 3 fish (200.5 ± 5.3 g) and seeded in triplicate wells. Cells were cultured for 3 days as described in 2.2.1.2 before adding 10 μg/ml of PGN or poly I:C into the cell culture well and the plate gently stirred for 10 s. The culture was incubated at 28 °C with 3 % CO_2 for 6 and 12 h to examine the time course response of target genes to PGN (TNF-α, IL-1β, IL-6, IL-10 and TGF-β) and poly I:C (TLR3, IRF3 and Viperin) before harvesting by pipetting and scrapping. After this first trial, the expeiment was repeated using 9 fish (198.5±11.2 g) that were collected for macrophage primary cell culture and cells were activated with PGN and poly I:C for 12 h. In this second experiment, PGN stimulated cells were used to determine transcription levels of TNF-α, IL-1β, IL-6, IL-10 and TGF-β, while poly I:C stimulated cells were used to determine transcription levels of IRF3, IL-10 and viperin, TLR2, TLR3, TLR8 and TLR9. The cell suspension of each well was transferred to an Eppendorf tube and cells separated by centrifugation at 1,500 x *g* for 5 min. Cells were then lysed with 1 ml of TRI Reagent (1 ml of TRI Reagent is sufficient for 5-10 x 10^6 cells). A mixture of cell and TRI Reagent was stored in -20 °C for RNA extraction process.

3.2.3.2 RNA extraction

Cells cultured in each well were preserved in TRI Reagent and stored in -20 °C. For RNA extraction, samples were left to defrost at the room temperature and 100 µl of 1-bromo-3-chloropropane was added. Samples were vortexed until the colour turn milky and incubated for 15 min at room temperature. For phrase separation, the mixture was centrifuged at 13,000 x *g* for 15 min at 4 °C. 400 µl of aqueous phrase was transferred to a new tube and 200 µl of isopropanol and 200 µl of RNA precipitation solution were added. The solution was mixed by inversion for 6-8 times and incubated at the room temperature for 1 h or -20 °C overnight. Samples were then centrifuged at 13,000 x *g* for 15 min at 4 °C. RNA pellets were washed with 1 ml of 75 % ethanol. Samples were incubated at room temperature for 5 min before centrifugation at 13,000 x *g* for 5 min, the ethanol discarded and the pellet dried by air-drying. Finally, an appropriate volume of DNAse/RNase free water was added for dissolution and stored at -70 °C. The RNA quality was measured with Nanodrop® ND-1000 (ThermoScientific, MA, USA).

3.2.3.3 cDNA synthesis

RNA was converted to a cDNA using the Precision nanoScript2 Reverse transcription kit (Primer Design, Chandlers Ford, UK). Based on the manufacturer's instructions, 1500 ng of RNA was used as a template for cDNA synthesis and the reaction was performed in a 0.2 ml tube. There were 2 steps in the reaction, in the first step; 1500 ng of RNA and 1 µl of Oligo-dT primers were added into 0.2 ml tube, then a final volume was made up to 10 µl with RNAase/DNAse free water. The mixture was heated at 65 °C for 5 min to allow the primers to anneal with the RNA template and then the tube was immediately transferred to ice. In the second step; a mixture was prepared with 5 µl of nanoscript2 buffer, 1 µl of 10 mM dNTP, nanoscript2 enzyme(reverse transcriptase) and 3 µl of RNAase/DNAse free water, the final volume was 10 µl. Then, 10 µl of the mixture was added to the tube on ice, mixed by pipetting and incubated at 42 °C for 20 min to synthesize the cDNA. After that the reaction was inactivated by heat at 75 °C for 20 min (Table3.2). The cDNA working solution was diluted 7.5 times with RNAase/DNAse free water and stored at -20 °C. Finally, the quality of cDNA was estimated by PCR with β-actin primers.

Table 3.2 A cDNA synthesis protocol

Component	1 reaction	
RNA template up to	9 µl	Incubated at 65 °C for 5 minutes
Primer (oligo dT)	1 µl	Then immediately on ice
Final volume	10 µl	
Nanoscript2 4X buffer	5 µl	Incubated at 42 °C for 20 minutes and
dNTP mix 10mM	1 µl	75 °C for minutes
RNAse/DNAse free water	3 µl	
Nanoscript2 enzyme	1 µl	
Final volume	10 µl	

3.2.3.4 Quantitative real-time PCR (qPCR)

An absolute qPCR was performed with Stratagene Mx3005P (Agilent Technology, CA, USA) to examine the level of mRNA expression by a comparison between a copy number of mRNA of target genes expressed in the macrophages and a copy number of standard plasmids containing the target amplicon. The qPCR was conducted in triplicate. The cDNA samples were amplified with 1 µl of 10 mM forward primer, 1 µl of 10 mM reverse primer, 10 µl of Luminaris Colour HiGreen qPCR master mix (ThermoScientific, MA, USA) and 6 µl of DNAse/RNAse free water to a final volume as 20 µl per reaction. The qPCR was conducted in 3 cycling steps; the first step is Uracil-DNA Glycosylase (UGD) pre-activation at 50 °C for 2 min, the second step is initial denaturation at 95 °C for 3 min and the third step is DNA synthesis consisting of denaturation at 95 °C for 15 s, annealing at various temperatures depending on primers for 30 s and then extension at 72 °C for 30 s. The DNA synthesis step was performed for 35 cycles then followed by melting curve analysis to verify the specificity of the PCR product at 95 °C for 1 min, 55 °C for 30 s and 95 °C for 30 s (Table 3.3). The copy number of the mRNA molecules was calculated according to a copy number of standard plasmid and cycle threshold value (Ct value) of each gene.

Table 3.3 Quantitative real-time PCR (qPCR) protocol

Component	1 reaction
Template	3 µl
10 mM forwards primer	1 µl
10 mM reverse primer	1 µl
Luminaris Colour HiGreen qPCR master mix	10 µl
DNAse/RNAse free water	5 µl
Final volume	20 µl

Step	Temperature	Time	
UGD pre-treatment	50 °C	2 min	
Initial denaturation	95 °C	3 min	
Denaturation	95 °C	15 sec	35 cycles
Annealing	56-61 °C	10 sec	
Extension	72 °C	10 sec	
Melting curve	95 °C	1 min	
	55 °C	30 sec	
	95 °C	30 sec	

3.2.3.5 Statistical analysis

Raw data of mRNA copies number obtained from qPCR were processed using Microsoft Excel 2013. Paired t-test was used to analyse the differences between copy number of stimulated mRNA and control mRNA after 12 h stimulation with a level of significance at p-value<0.05. whereas, ANOVA and multiple comparison were used in comparioson a level of mRNA transcription between different time points of cell stimulation. Graphs were generated using GraphPad Prism version 6.0 (San Diego, CA, USA).

3.3 Results

3.2.1 Molecular tool development

This study aimed to characterise the interaction between Pathogen Associated-Molecular Patterns (PAMPs) and Pattern Recognition Receptors (PRRs), which is a critical function of innate immunity in the host. mRNA sequences of cytokine and signalling genes of Nile tilapia were analysed in these experiments. mRNA sequences of 11 innate immunity-related genes were selected from the NCBI database and gene-specific primers were successfully designed showing a single band on RT- PCR (Fig 3.1) and a single peak on qPCR. There were 5 genes related to antibacterial response; TNF-α, IL-1β, IL-6, IL-10 and TGF-β, 2 genes related to antiviral response; Viperin and IRF3 and 4 receptor genes; TLR2,

TLR3, TLR8, TLR9. Moreover, β-actin, a housekeeping gene, was analysed to use for cDNA quality measurement (Table 3.4). Cloning of these 11 genes was done. The plasmid was extracted and the target fragment confirmed by sequencing with 100% identity to mRNA template gene. The sequences of PCR products are available in appendix 2. Moreover, a series dilution of selected gene plasmids was done and performed qPCR to generate a standard copy number for an absolute qPCR, Ct-value and copy number of the genes were shown in appendix 3.

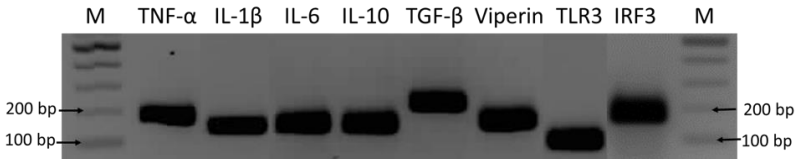


Figure 3.1; Agarose gel electrophoresis image of colony PCR, a PCR was performed with 2 µl of *E.coli* and 1 µl of 10 µM primers. The PCR product was measured with 1.5% of agarose gel and visualized under the UV light. The PCR product size of each primers range between 230 bp to 108 bp and M is a DNA ladder.

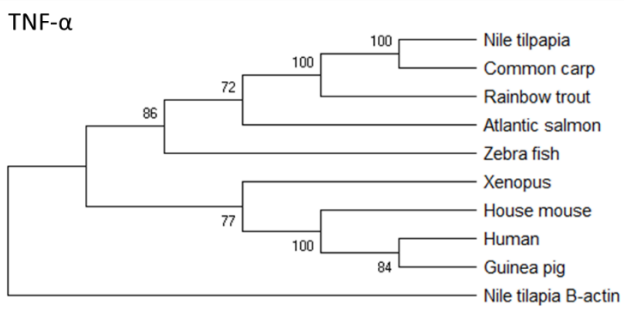
Table 3.4 List of gene-specific primers with their product size, annealing temperature, and accession number of the gene

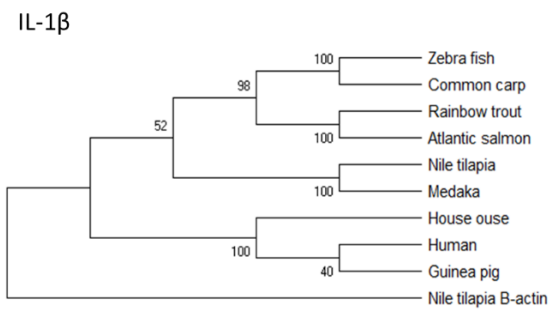
Genes	Sequence	Product Size	T_a	Accession number
TNF-α	>F CAGGATCTGGCGCTACTCAG	184 bp	60 ˚C	NM_001279533.1
	>R TAGCTGGTTGGTTTCCGTCC			
IL-1β	>F TGAGAGCCTACTTTAGGATTCTGC	150 bp	59 ˚C	XM_005457887.2
	>R GCGGCTATTACAACCAATGCT			
IL-6	>F CTGAGTGAGGGGAAAAGAGC	148 bp	61 ˚C	XM_019350387.2
	>R AGGAGTGTCAAAACCATCCAG			
IL-10	>F CTCAGATGGAGAGCAGAGGTC	134 bp	60 ˚C	KP645180.1
	>R CTTGATTTGGGTCAGCAGGT			
TGF-β	>F GAGATCCCTGCCAACTTGCT	230 bp	60 ˚C	NM_001311325.1
	>R TCCCCGACGTTACTCCGTAT			
Viperin	>F ATCAACTTCTCTGGCGGA	161 bp	56 ˚C	XM_003453237.3
	>R AGATAGACACCATATTTCTGGAAC			
IRF3	>F GGTACGACACATCAGCGTGC	183 bp	60 ˚C	XM_005448320.3
	>R CTGGCAACATAGAGCAGCAGTA			
TLR2	>F TCTGGGCTATCCTTCCCCAA	221 bp	60 ˚C	XM_013264298.3
	>R TCGCAGATGTAGCTGTCCAC			
TLR3	>F CTGTCCGTCACTCCGAAACA	108 bp	59 ˚C	XM_003449728.4
	>R CCGGGATTGATCTGCGCTAT			
TLR8	>F TCTGAGTGGGTGATGAGCA	137 bp	61 ˚C	XM_019352831.2

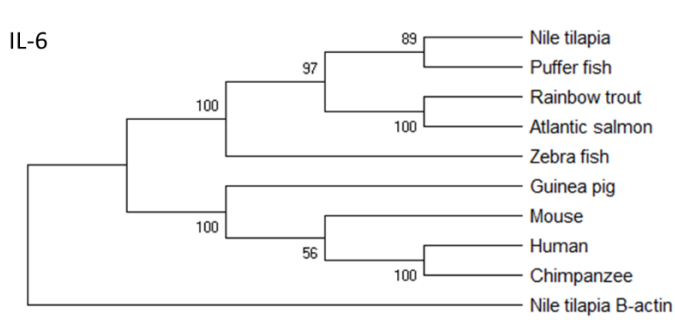
TLR9	>R TGTACTGGATGCTCTGGGTG > ACCTTCCTGGACCTCAGTCA > TGGCATGCAGGGTGAGATTT	178 bp	60 ˚C	XM_005477981.4
β-actin	>F GCTACTCCTTCACCACCACAG >R CGTCAGGCAGCTCGTAACTC	144 bp	61˚C	KJ126772.1

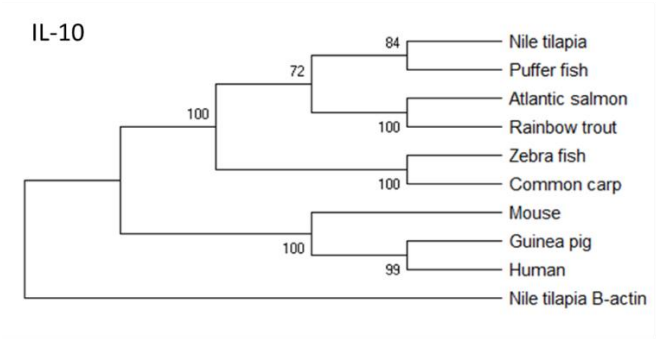
3.2.2 Evolutionary relationships

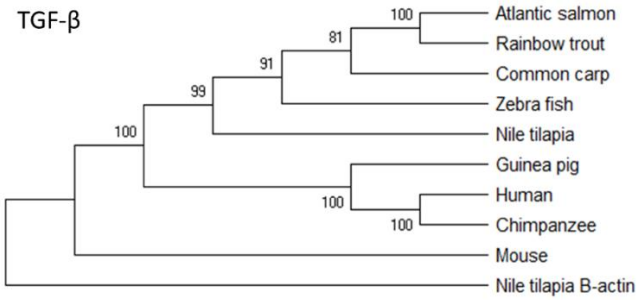
The phylogenetic tree of 11 target genes was constructed to study an evolutionary relationship between Nile tilapia and other vertebrate species. The similarity between taxa was measured using UPGMA and phylogeny of each gene was tested with 500 bootstrap replications. There were 2 main groups of taxa obtained from a database; teleost (common carp, zebrafish, rainbow trout, Atlantic salmon, medaka and pufferfish) and mammalian (human, chimpanzee, guinea pig, and house mouse). Overall, the phylogenetic tree of each gene presented 2 major clades which separated teleost from mammalian which 99-100 bootstrap score. In mammalian, the innate immunity-related genes of human were closely related to a chimpanzee with 100 bootstrap score followed by mouse and guinea pig. In terms of teleost, minor clades were distingue by order of organisms. They were members of order Cypriniformes (common carp and zebrafish), Salmoniformes (Atlantic salmon and rainbow trout), Cichliformes (Nile tilapia), Beloniformes (medaka) and Tetraodontiformes (pufferfish). The multiple alignments of antiviral related genes (IRF3 and viperin) sequence of Nile tilapia was homologous to medaka (100 scores). Receptor genes sequences; TLR2, TLR3 and TLR8 were closely related to Atlantic salmon and rainbow trout (100, 99 and 71 scores, respectively), while TLR9 sequence showed high homology to pufferfish (99 scores). For cytokine genes, IL-6 and IL-10 sequence of Nile tilapia were similar to puffer fish (89 and 84 scores) and, IL-1β sequence was homologous to medaka (100 bootstrap score) while TNF-α sequence of Nile tilapia and common carp were homologous with 100 bootstrap score. However, the relationship between TGF-β of Nile tilapia and other taxa was unclear (Fig. 3.2). A multiple sequences alignment and conserved region of TGF-β of Nile tilapia and other teleost was presented in Fig.3.3.

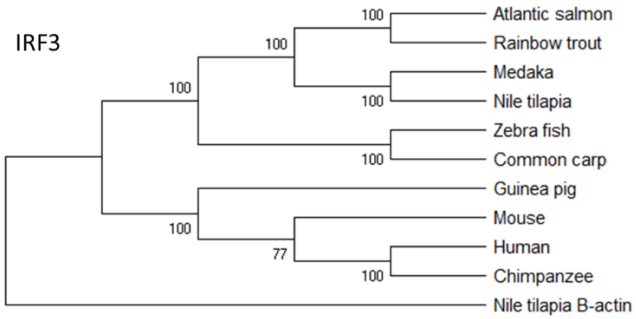

TNF-α
Nile tilpapia
Common carp
Rainbow trout
Atlantic salmon
Zebra fish
Xenopus
House mouse
Human
Guinea pig
Nile tilapia B-actin
100
100
72
86
77
100
84

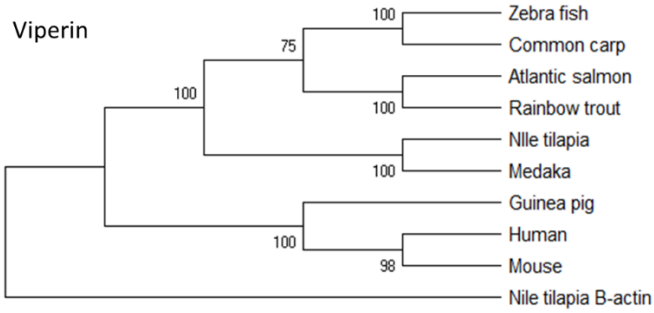

IL-1β
Zebra fish
Common carp
Rainbow trout
Atlantic salmon
Nile tilapia
Medaka
House ouse
Human
Guinea pig
Nile tilapia B-actin
100
98
52
100
100
100
40


IL-6
Nile tilapia
Puffer fish
Rainbow trout
Atlantic salmon
Zebra fish
Guinea pig
Mouse
Human
Chimpanzee
Nile tilapia B-actin
89
97
100
100
100
56
100


IL-10
Nile tilapia
Puffer fish
Atlantic salmon
Rainbow trout
Zebra fish
Common carp
Mouse
Guinea pig
Human
Nile tilapia B-actin
84
72
100
100
100
100
99


TGF-β
Atlantic salmon
Rainbow trout
Common carp
Zebra fish
Nile tilapia
Guinea pig
Human
Chimpanzee
Mouse
Nile tilapia B-actin
100
81
91
99
100
100
100


IRF3
Atlantic salmon
Rainbow trout
Medaka
Nile tilapia
Zebra fish
Common carp
Guinea pig
Mouse
Human
Chimpanzee
Nile tilapia B-actin
100
100
100
100
100
100
100
77
100


Viperin
Zebra fish
Common carp
Atlantic salmon
Rainbow trout
NIle tilapia
Medaka
Guinea pig
Human
Mouse
Nile tilapia B-actin
100
75
100
100
100
100
98

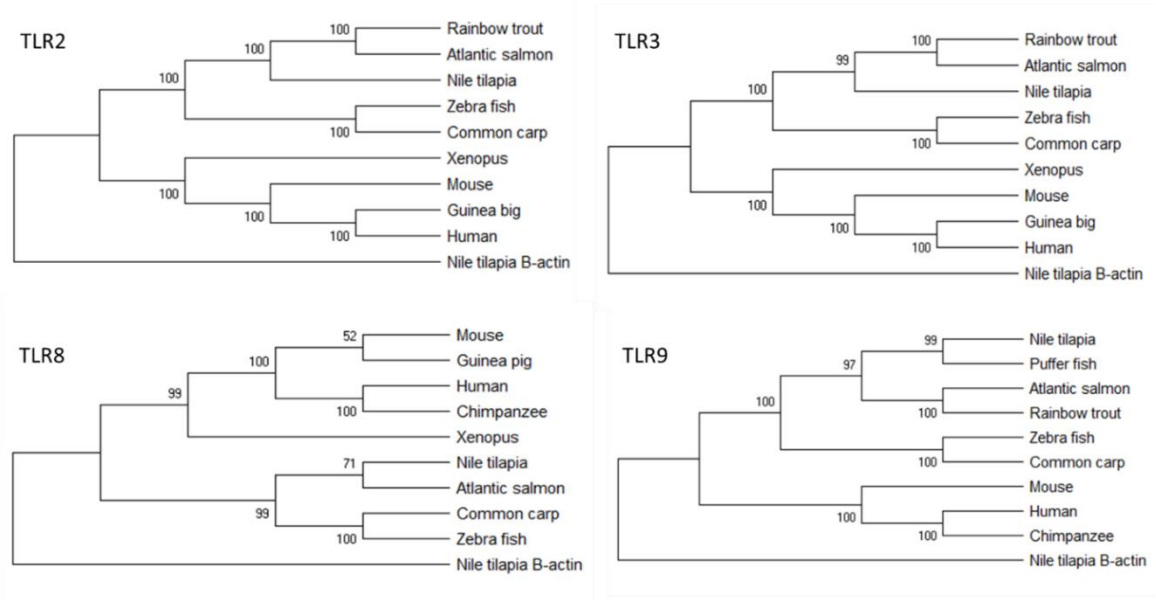


Figure 3.2 An unrooted phylogenetic tree of selected genes. The tree was constructed using mRNA sequences multiple alignments and the UPGMA method with the MEGAX program. Node values represent the percentage of bootstrap confidence derived from 500 replicates. β-actin of Nile tilapia was used as an out group of each tree. The accession number for each sequence was attached in appendix 2.

```
Tilapia     GACCTCGACTCATTACCTGGCATCTCGCTTTGTCACAAACACGCTGAAGGACAAATGGCT  694
Rainbow     ------------------------------------CAAGGAATGGGCCAATCGCTGGGT   24
Common      GAACAAGTCACGCTACCTGGAATCACGCTTTATTCCCAACCAA------AGAAAGTGGCT  727
Zebrafish   GGATCAGGCTCGATATCTGGGAACTCGCTTTGTCTCCAAGGACTTGTCCAACCGCTGGCT  704
Atlantic    AGATAAGGCACGTTACATGAAGTCCCATTTTATCTCAAAGGAATGGGCCAATCGCTGGAT  415
                                          **                  *** *

Tilapia     GTCCTTTGGTGTCACTGAACCTCTGCAGACCTGGCTCCAAGGGAATGAGAATGAACAGAA  754
Rainbow     GTCCTTTGATGTAACACAGACTCTGAATGAGTGGCTGCAAGGGGCTGGAGAGGAGCAGGG   84
Common      ATCGTTTGATGTGACGCAGACCTTGAAAGACTGGCTGCAGAGAAGTGAGGCAGAGCAAGG  787
Zebrafish   CTCATTTGACGTGAAACAGACAATGATAGAATGGCTGCAGGGTTCAGAAGATGAAGAAAC  764
Atlantic    CTCCTTTGATGTTACACAGACTCTGAGGGAGTGGCTGCAAGGGGCTGGAAAGGAGCAAGG  475
             ** ****  ** *   *  *  **      ***** **  *    *     **  *

Tilapia     GTTTGAACTTCGGCGGTACTGTGAATGCGGCAATAACGA---------TGATACGTTAAG  805
Rainbow     ATTCCAATTGAAGTTGCCTTGTGATTGTGGGAAAC---------CAATGGAGGAATTCCG  135
Common      ATTTCAACTGAAGATGGCGGATAACTGTGATCCTC------------AGAAGACATTTCA  835
Zebrafish   CTTGGAGCTGAGGTTATACTGCGACTGTAAAGCAAACCAGCAGAGCACGGATAAGTTCCT  824
Atlantic    ATTCCAATTGAAGTTGCCTTGTGATTGTGGGAAAA---------CAATGGAAGAATTCCG  526
             **  *  *   *          * **                  *    **

Tilapia     TTTTAGCATCTCTGGGACTATGAGCAGGAGGGGAGACACTAAAGACTTACAGAAGCTGAA  865
Rainbow     CTTCAAAATATCAGGGATGAACAAGCTGAGGGGAGATACAGAAACACTAGCCATGAAAAT  195
Common      ACTCAAAATACCAGGTTTGGTTCTTGTAAGAGGTGATACAGAAACCTTAGCAGTAAAT--  893
Zebrafish   CTTCACTATATCAGGGCTAGACAAGCAGAGAGGTGACACCGCTGGTCTGGCAGATATG--  882
Atlantic    CTTTGAAATAGCAGGGATGAACAAGCCCARGGGAGATAAAGAAACTCTCTTCATGAAC--  584
              *    **  * **              *  ** ** *        *

Tilapia     CCAGCAGCTACCCTACATCTTTACCATGTCCATCCCT---AAAACCAACCACTCTAAGAG  922
Rainbow     GCCATCAAAGCCTCACATTTTACTGATGTCACTTCCTGTGGAACGCCACAGCCAGCTTAG  255
Common      -ATGCCAAGGCCTCACATTTTGGTAATGTCACTTCCTCTTGATGGCAACAACTCATCAAA  952
Zebrafish   -ATGGTGAAGCCGTACATTTTGGCTTTATCTTTGCCTTCAAATGGCAACTCTCTTGCATC  941
Atlantic    -ATGCCAAAGCCTCACATTTTACTGATGTCACTTCCTGTGGAACGCCACAGCCAGCTTAG  643
                    **  **** **     * **  * ***    *   * **

Tilapia     TTTACGCACAAAACGTTCCACTGACACAACGGACAGCTGCGGCACCCAATCACACAACTG  982
Rainbow     CTCTCGGAAGAAACGACAAACCACTACTGAAGAGATCTGCTCAGATAAATCGGAGAGTTG  315
Common      ATCTCGCAGAAAACGTCAAACTGAAACAGATCAAGTTTGCACCGATAAGTCTGACGGCTG 1012
Zebrafish   CGTCCGCAAGAGACGAGCTGTTGGCACCGATGAAACATGTGATGAAAAAACGGAGACCTG 1001
Atlantic    CTCTCGGAAGAAACGACAAACCACTACTGAAGAGATCTGCTCAGACAAATCGGAGAGTTG  703
              ** *  * ***          **    *    **        *  *  *    **

Tilapia     CTGTTTGAAGAAACTGTACATTGACTTCAGGAAAGATCTAGGATGGAAGTGGATCCATAA 1042
Rainbow     CTGTGTGCGAAAACTTTACATTGACTTCCGTAAGGACCTGGGCTGGAAGTGGATCCATGA  375
Common      CTGTGTGAGAAGTCTGTACATTGACTTCCGCAAAGACCTGGGCTGGAAGTGGATACATGA 1072
Zebrafish   CTGTATGCGCAAGCTTTACATTGACTTTCGGAAAGATCTGGGTTGGAAGTGGATCCACAA 1061
Atlantic    CTGTGTGCGAAAGCTTTACATTGACTTCCGTAAGGACCTGGGCTGGAAATGGATCCATGA  763
              **** **   *  ** ***********  * ** ** ** ** ***** ***** **  *

Tilapia     GCCAACGGGTTACTATGCTAACTACTGCATGGGGTCCTGCACCTATATCTGGGATGCCGA 1102
Rainbow     ACCCACTGGCTACTTTGCTAACTACTGCATCGGCCCCTGCACCTATATATGGAACACAGA  435
Common      ACCTTCTGGTTATTATGCCAACTATTGCACTGGCTCTTGCTCTTTCGTCTGGACTTCAGA 1132
Zebrafish   GCCCAAGGGGTACTTTGCCAACTACTGCATGGGGTCCTGCACCTACATCTGGAATGCTGA 1121
Atlantic    ACCCACTGGCTACTTTGCTAACTACTGCATGGGCCCCTGCACCTATATATGGAACACAGA  823
             **    ** ** * *** ***** ****  **  * *** * *   * ***    * **

Tilapia     AAACAAATATTCTCAGATTCTGGCACTGTACAAACATCATAACCCAGGAGCTTCTGCCCA 1162
Rainbow     AAACAAGTATTCCCAGGTACTGGCTCTGTATAAGCACCACAACCCCGGAGCCTCTGCCCA  495
Common      AAATAAGTACTCACAGGTTCTTGCGCTATATAAGCATCACAATCCTGGTGCATCTGCTCA 1192
Zebrafish   GAACAAATACTCACAGATATTAGCCTTGTACAAACACCACAACCCTGGTGCCTCGGCTCA 1181
Atlantic    GAACAAGTACTCCCAGGTACTGGCTCTGTATAAGCACCACAATCCTGGAGCCTCTGCCCA  883
             ** ** ** ** *** *  * **  * ** ** ** ** ** ** ** ** ** ** **

Tilapia     GCCCTGCTGTGCGCCACAGACACTAGAGCCACTGCCAATCATCTACTATGTGGGGAGGCA 1222
Rainbow     CCCCTGCTGTGTTCCACAGGTCTTGGAGCCCCTCCCCATTATCTATTATGTGGGGAGACA  555
Common      ACCCTGCCGTGTACCTCAAGTTCTAAACCCACTGCCCATCTTTTACTATGTGGGCCGGCA 1252
Zebrafish   GCCATGCTGTGTACCCGCAATCCTTGACCCTCTGCCAATTCTTTATTACGTGGGAAGGCA 1241
Atlantic    ACCTTGCTGTGTTCCACAGGTCTTGGAGCCCCTACCCATTCTCTACTATGTGGGTAGACA  943
             ** *** ***  **        *  * ** ** ** **  * ** ** *****  * **

Tilapia     ACACAAGGTCGAGCAGCTGTCCAATATGATCGTGAAGTCCTGCAAGTGTAGCTAA-----  1277
Rainbow     ACACAAGGTGGAGCAGGTGCCCAATATGATTGTTATGTCCTGCAGGTGTAGCTAGAATTA  615
Common      ACATAAGGTAGAACAACTGTCAAATATGATTGTGAAGACCTGCAAGTGTTGCTGAAAGCC 1312
Zebrafish   ACACAAGGTGGAGCAATTGTCCAACATGGTGGTGAGGAACTGCAAGTGCAGTTAAGAGTT 1301
Atlantic    ACACAAGGTGG-------------------------------------------------  954
            *** ***** *
```

Figure 3.3 Multiple sequence alignment of TGF-β of Nile tilapia and teleost using ClustalW. Stars denote a conserve region among sequences of taxa.

3.2.3 PAMP-PRR interactions

In the first experiment, macrophages of Nile tilapia were stimulated with 10 µg/ml of PGN or 10 µg/ml of poly I:C and then incubated at 28 °C for 6 and 12 h. Stimulated and control (non-stimulated) cells were harvested and RNA was extracted to investigate the gene expression of targeted genes. RNA was converted to cDNA and cDNA quality was assessed using β-actin primers. β-actin primers produced a single clear band with a product size of 144 bp and the density of each bands was mostly similar (Fig 3.4) suggesting that the cDNA samples were of good quality and suitable to perform qPCR analysis.

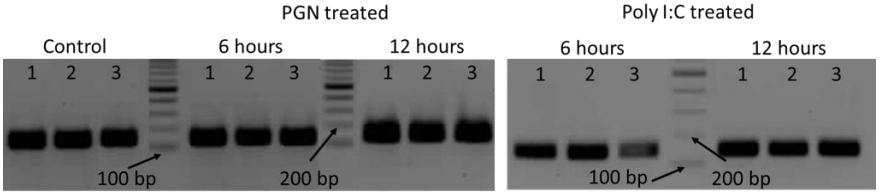


Figure 3.4 Agarose gel electrophoresis images of cDNA with β-actin primers. Macrophage primary cell stimulated with 10 µg/ml of PGN and 10 µg/ml of Poly I:C for 6 and 12 hours. Control is a non-stimulated cell. Product size of β-actin primers is 144 bp.

After stimulation with PGN, pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) presented upregulation trend of mRNA. Copy number of TNF-α mRNA gradually increased after 6 and 12 h of stimulation whereas that of IL-1β was stable at 6 and 12 hours post-activation. However, a contrasting trend was found in IL-6, which mRNA increased after 6 h of treatment and then slightly decreased at 12 h. In terms of anti-inflammatory cytokines (IL-10 and TGF-β), the amount of IL-10 mRNA was down-regulated at 6 h then highly upregulated at 12 h (the highest point). While the mRNA of TGF-β showed the lowest point at 6 h and it rose again at 12 h after treatment but still lower than control. For poly I:C treated cells, the mRNA of IRF3 and Viperin mRNA significantly increased after 6 h post-stimulation ($p<0.05$), then they slightly dropped after 12 h but still higher than that of control. While, TLR3 mRNA showed a gradual increase from 6 to 12 h although the increase was not significant ($p>0.05$).

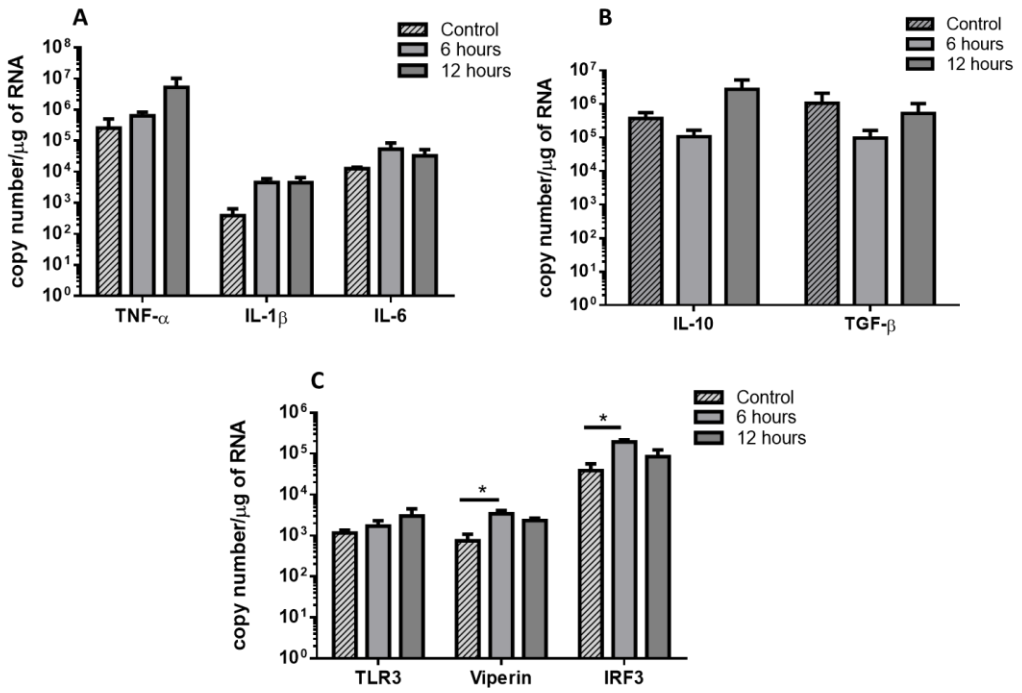


Figure 3.5 The level of mRNA transcribed at 6 h and 12 h after stimulation. A: a copy number of pro-inflammatory cytokines treated with 10 μg/ml of PGN, B: a copy number of anti-inflammatory cytokines treated with 10 μg/ml of PGN, C: a copy number of antiviral related genes treated with 10 μg/ml of poly I:C. The experiments have been done in 3 biological and technical replication. ANOVA and multiple comparisons was tested with a level of significance; $P<0.05$. Stars above bar denote results significantly different.

After a time course trial, the macrophage primary cell stimulation experiment was repeated again. In this experiment, macrophages were incubated with 10 μg/ml of PGN or 10 μg/ml of poly I:C for 12 h before harvested. Moreover, the number of of Nile tilapia was increased to 9 fish/experiment and another 3 receptor genes were analysed; TLR2, TLR8 and TLR9. Macrophages stimulated with PGN were used to determined the transcription level pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), anti-inflammatory cytokines (IL-10 and TGF-β), whereas, the poly I:C stimulated cells were used to determined the transcription level of antiviral related genes (IRF3, IL-10 and viperin), and receptor genes (TLR2, TLR3, TLR8 and TLR9) by qPCR.

The quality of cDNA was measured by amplified with β-actin primers which appeared a single clear band of 144 bp amplicon (Fig. 3.6). The transcription level of cytokines was measured after treated with PGN for 12 h. However, there were some technical error after qPCR, therefore only 6 samples were used in statistical analysis. Results reveal an upregulation trend of pro-inflammatory cytokines mRNA after PGN treatment. IL-6 showed a significantly increasing amount of mRNA ($p=0.0239$), which

was the largest increasy by approximately 43 times, followed by TNF-α and IL-1β with 4.4 and 2.5 times compared with control (non-stimulated cells). For anti-inflammatory cytokines, the mRNA of TGF-β showed significantly down-regulation (p=0.0287) when compared with control mRNA. In contrast, the level of IL-10 mRNA presented upregulation with 4.5 times higher than control (Fig 3.7).

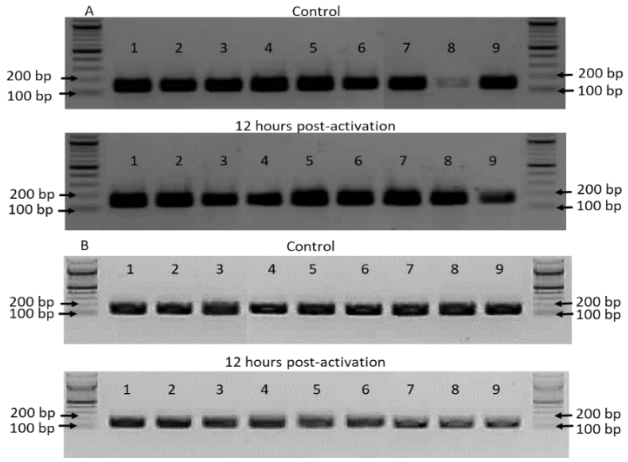


Figure 3.6 Agarose gel electrophoresis images of cDNA with β-actin primers. A: macrophage primary cell stimulated with 10 µg/ml of PGN for 12 hours and control (non-stimulated cell) B: macrophage primary cell stimulated with 10 µg/ml of poly I:C for 12 hours and control (non-stimulated cell). Each gels contain 9 samples and product size: 144 bp

In terms of poly I:C stimulation, the mRNA of all antiviral related genes; IRF3, IL-10 and Viperin showed an upregulation of mRNA transcription. The mRNA transcribed of IRF3 and Viperin significantly increased (*P*-value were 0.01 and 0.0028) when compared with mRNA of control cells. However, an increasing amount of IL-10 mRNA was found with approximately 1.9 times. In the case of receptor genes (TLR2, TLR3, TLR8 and TLR9), the level of mRNA transcription was determined when cells were stimulated with poly I:C. Only TLR3 presented increasing amount of mRNA after stimulation by approximately 2.3 times compared to control, whereas TLR2 was downregulated (-2.2 fold change), while no changes were observed for TLR8 and TLR9 (Fig.3.8). A statistical analysis is available in appendix 5.

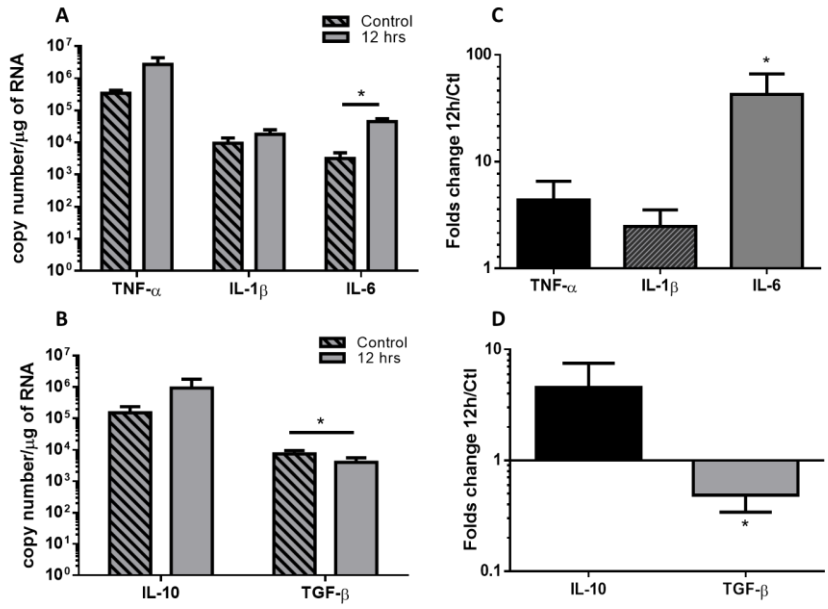


Figure 3.7 mRNA transcription levels of cytokines after 12 h stimulation with 10 μg/ml of PGN. A: a copy number of pro-inflammatory cytokines mRNA, B: a copy number of anti-inflammatory cytokines mRNA, C: a fold change difference of pro-inflammatory cytokines mRNA, D: a fold change difference of anti-inflammatory cytokines mRNA. The data was analyzed using t-test with a level of significance; *P*<0.05 (n=6). Stars above bar denotes results significantly different.

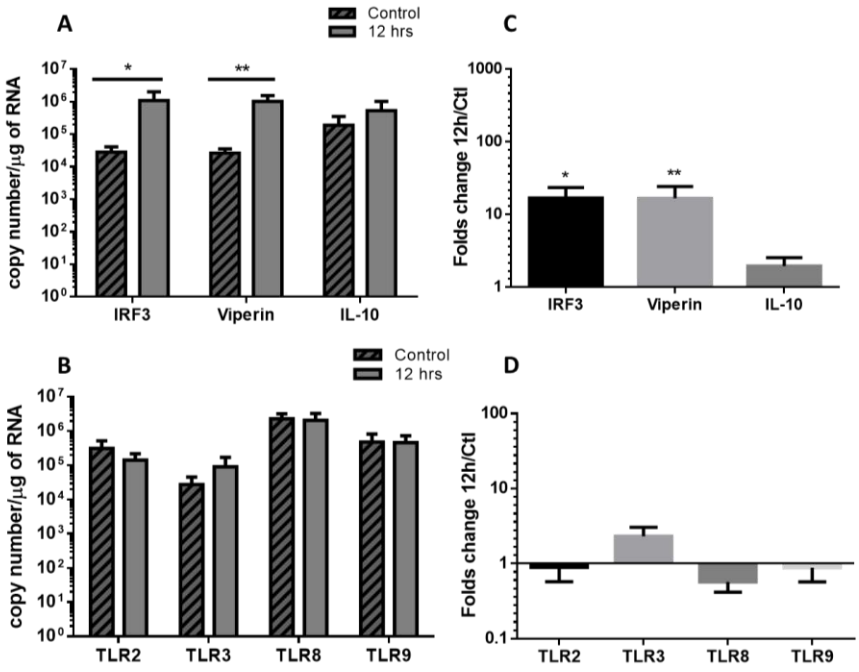


Figure 3.8 A comparison of antiviral related genes and receptor genes expression between control and 12 hours after stimulation with 10 μg/ml of poly I:C. A: a copy number of antiviral related genes mRNA, B: a copy number of receptor genes mRNA, C: a fold change difference of antiviral related genes mRNA, D: a fold change difference of receptor genes mRNA. The data was analyzed using t-test with a level of significance; *P*<0.05 (n=6). Stars above bar denotes results significantly different.

3.4 Discussion

3.4.1 Evolutionary relationship

In this study, a phylogenetic tree of selected genes was constructed using mRNA sequences multiple alignments and the UPGMA method with the MEGAX program to determine the evolutionary relationship and the conservation of these selected genes between Nile tilapia and other taxa. The mRNA sequences of different organisms were obtained from the database were mammalian (human, chimpanzee, guinea pig, and house mouse) and teleost (common carp, zebrafish, rainbow trout, Atlantic salmon, medaka and pufferfish). Sample organisms were selected by their habitat and genetic background to determine the conservation of the genes. The phylogenetic tree of selected genes revealed 2 major clades between mammalian and teleost. Most of the selected genes have been well characterised in mammalian species particularly in human. The phylogeny of the genes showed a homologous between human and chimpanzee with 100 bootstrap score, as these 2 species diverged from the same common ancestor (Waterson et al 2005), followed by mouse and guinea pig. In teleost, the evolutionary conservation of the gene diverged between the orders of taxa; Salmoniformes (Atlantic salmon and rainbow trout) and Cypriniformes (common carp and zebrafish) were always grouped in the same minor clades except in TNF-α and TGF-β. In terms of Nile tilapia (Cichliformes), IL-6, IL-10 and TLR9 were homologous to pufferfish (Tetraodontiformes), and IRF3, Viperin and IL-1β were homologous to medaka (Beloniformes). TLR2, TLR3 and TLR8 showed high homology to Atlantic salmon and rainbow trout, while TNF-α was homologous to common carp.

According to the phylogenetic classification of teleost (Betancur-R et al 2017), by the evolutionary period, common carp, Nile tilapia, medaka and pufferfish emerged approximately 100 million years ago in Cretaceous periods. Nile tilapia, medaka and pufferfish diverged from their common ancestor at the same period, whereas they shared a common ancestor with common carp back to 250 million years ago. As a result, there were 6 out of 11 genes (IL-6, IL-10, TLR9, IRF3, Viperin and IL-1β) of innate immunity gene of Nile tilapia show sequence homology compared to pufferfish and medaka, while 1 gene (TNF-α) was homologous to common carp. In terms of Salmoniformes (Atlantic salmon and rainbow trout) is the newest order which diverged 50 million years ago and shared a common ancestor with Nile tilapia (Cichliformes) at 200 million years ago. Therefore, there were 3 homolog genes (TLR2, TLR3 and TLR8) between Nile tilapia and Atlantic salmon and rainbow trout. The evolutionary and

conservation of TLR have been reported in many studied (Table 3.7). It was found that 18 TLRs have been found in teleost but the fish have their specific TLR depends on species (Zhang et al 2013). The loss or expansion of TLRs was an effect of host-intrinsic factor or environmental changes which force an evolutionary of immunity (Solbakken et al 2017). According to a study of Solbakken et al (2017) demonstrated that TLR9 was well conserved in teleost and present multiple copies of gene in many species followed by TLR3 and TLR8, while TLR2 was absent in several species of Gadiformes. Moreover, the evolution of TLR9 have been studied by Zhu, Z. et al (2013) illustrate a conservation of TLR9 in Perciformes and the evolution was drove by local environment which possessed different pathogens to the organisms. The result of this study appears to support an evolutionary conservation of innate immunity genes of teleost. Besides, the characterization and conservation of these target genes have been report in many studies as described in Table 3.5.

Table 3.5 Example of innate immunity genes which have been studied in teleost fish.

Genes	Organism	Ref.
TNF-α	Rainbow trout (*Onchorhynchus mykiss*)	(Hong, S. et al 2013)
	Common carp (*Cyprinus carpio*)	(Saeij et al 2003)
	Channel catfish (*Ichtalurus punctatus*)	(Zou et al 2003)
IL-1β	Japanese flounder (*Paralichthys olivaceus*)	(Taechavasonyoo et al 2013)
	Salmonids	(Husain et al 2012)
	Atlantic cod (*Gadus morhua*)	(Seppola et al 2008)
IL-6	Orange-spotted grouper (*Epinephelus coioides*)	(Chen, H. et al 2012)
	Large yellow croaker (*Larimichthys crocea*)	(Zhu, Q. et al 2016)
	Zebrafish (*Danio rerio*)	(Varela et al 2012)
IL-10	Golden pompado (*Trachinotus ovatus*)	(Peng et al 2017)
	Goldfish (*Carassius auratus*)	(Grayfer et al 2011)
	Puffer fish (*Fugu rubripes*)	(Zou et al 2003)
	Atlantic cod (*Gadus morhua*)	(Seppola et al 2008)
TGF-β	Goldfish (*Carassius auratus*)	(Haddad et al 2008)
	Grass carp (*Ctenopharyngodon idella*)	(Yang, M. and Zhou 2008)
IRF3	European eel (*Anguilla anguilla*)	(Huang, B. et al 2014)
	Japanese flounder (*Paralichthys olivaceus*)	(Hu et al 2011)
	Miiuy croaker (*Miichthys miiuy*)	(Shu et al 2016)
	Nile tilapia (*Oreochromis niloticus*)	(Gu et al 2016)

Viperin	Nile tilapia (*Oreochromis niloticus*)	(Lee et al 2013)
	Large yellow croaker (*Larimichthys crocea*)	(Zhang, Jianshe et al 2018)
	Mandarin fish (*Siniperca chuatsi*)	(Sun and Nie 2004)
TLR2	Turbot (*Scophthalmus maximus*)	(Liu et al 2016)
	Common carp (*Cyprinus carpio*)	(Fink et al 2016)
	Gibel carp (*Carassius auratus gibelio*)	(Fan et al 2018)
	Rainbow trout (*Oncorhynchus mykiss*)	(Brietzke et al 2016)
TLR3	Japanese flounder (*Paralichthys olivaceus*)	(Hwang et al 2012)
	Large yellow croaker *(Pseudosciaena crocea*)	(Huang, X. et al 2011)
	Orange-spotted grouper (*Epinephelus coioides*)	(Lin, K. et al 2013)
	Sea perch (*Lateolabrax japonicus*)	(Wang, P. et al 2018)
	Spiny eel (*Mastacembelus armatus*)	(Han et al 2017)
TLR8	Large yellow croaker (*Pseudosciaena crocea*)	(Qian et al 2013)
	Rainbow trout (*Oncorhynchus mykiss*)	(Palti et al 2010)
	Turbot (*Scophthalmus maximus*)	(Dong, X. et al 2016)
TLR9	Turbot (*Scophthalmus maximus*)	(Dong, X. et al 2016)
	Golden pompano (*Trachinotus ovatus*)	(Wei, Y. et al 2017)
	Japanese flounder (*Paralichthys olivaceus*)	(Takano et al 2007)

3.4.2 Response of innate immune system to bacterial infection

This study aims to examine the response of macrophages to bacterial infection in Nile tilapia via stimulation of macrophage primary cell culture with PGN. Cytokines are cell signalling, mainly synthesized by an immune cell as macrophages and T-cell, act as regulators of host responses to infection, inflammation (Dinarello 2000) and initiating a defense response by being a mediator between the innate immune system and adaptive immune system. The selected genes were categorized into 2 groups; pro-inflammatory (TNF-α, IL-1β and IL-6), triggering by PAMPs to initiate the innate immune response of the host, and anti-inflammatory (IL-10 and TGF-β) activate to reduce inflammation and promote healing stage by inhibiting pro-inflammatory cytokines activity. The level of genes expression was discriminated by the copy number of mRNA transcribed of the gene compared between activated cell and control cell using an absolute qPCR method.

Bacteria have specific components generally present on their cell wall; lipopolysaccharide (LPS) found in Gram-negative bacteria and peptidoglycans (PGNs) found in both Gram-negative and Gram-positive bacteria, could be recognized by the immune system of the host. Peptidoglycan (PGN) is an essential structural element in the bacterial cell composed of glycan chain cross-linked with short peptides (Vollmer et al 2008) which is a thin layer cover gram-negative bacteria whereas many times layer thicker in gram-positive bacteria. LPS are extensively used as a stimulation reagent for triggering immunity cell of a host in vitro in mammalian but not much effective in fish. In some cases, fish become tolerant to LPS due to a chronic exposure to the pathogen in their living environment (Forlenza et al 2011). LPS tolerance also found in mice after repeatedly treat with LPS (Erroi et al 1993).

Moreover, it has been found that the activation of pro-inflammatory cytokines genes activated with crude LPS was a result of PGN contamination, as the crude LPS contained PGN, nucleic acid and lipoprotein, but ultrapure LPS could not trigger pro-inflammatory cytokines genes (MacKenzie et al 2010). Besides, an absence of several of LPS recognition receptors (LBP, CD14, MD-2 and TICAM2) in fish families are reported (Iliev et al 2005), especially TLR4 which absent in several species of fish but present in zebrafish. However, recent study reported that caspy2 served as an intracellular LPS receptor in zebrafish (Yang et al 2018). Interestingly, a study of an innate immune response in Atlantic cod revealed that ultrapure PGN but not LPS triggered the innate immune response of Atlantic cod through NLR receptor due to the fish has lost many of TLRs families during it evolutionary (Lin et al, manuscript under review). It suggests that due to a potentially high pathogenic pressure in the extracellular environment, the fish rely more upon in cellular PAMP perception and this advocate an effective of PGN. Therefore, in this experiment, an ultrapure PGN, obtained from E.coli K-12, was used to examine its effect on inflammatory cytokines of Nile tilapia via the macrophage primary cell culture.

Result revealed that PGN triggered an accumulation of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) at 6 hours post-stimulation and the level of pro-inflammatory cytokines mRNA still presented a higher than that of control at 12 hours post-activation. For IL-6, the accumulation of mRNA was dropped after 6 hours. However, at 12 hours, it has 43 times of mRNA higher than that of control, which was the largest variation of all. In terms of anti-inflammatory cytokines (IL-10 and TGF-β), the trend of genes expression was different from pro-inflammatory cytokines, as the level of mRNA accumulation was dropped at 6 hours post-activation and then increased at 12 hours post-activation.

However, the amount of TGF-β mRNA was still lower than that of control, whereas the amount of IL-10 mRNA was the highest at 12 hours.

Pro-inflammatory cytokines; TNF-α, IL-1β and IL-6 are mainly synthesized in ER of the macrophages and play as endogenous pyrogen to induce fever and production of acute-phase protein. TNF-α and IL-1β are synthesized and released to the extracellular membrane as a processor which response at the first stage of infection or stress and have a critical role in triggering a cytokines cascade (Srinivasan et al 2017) and increase the gene expression in autocrine loop manner (Duque and Descoteaux 2014). A study of Roher et al (2011), reported a rapid secretion of TNF-α after LPS or zymosan stimulation which was pre-formed peptides of TNF-α, while the gene has delay expressed after the stimulation. Besides, it was suggested that TNF- α was a potent mediator of inflammation as it has been reported that suppression of TNF- α in cultures of synovial cells from rheumatoid arthritis (RA) patients inhibited the expression of IL-1 and other pro-inflammatory cytokines (Brennan et al 1989; Tseng et al 2018). Apart from being inflammation mediator, TNF- α also function in cell survival signaling pathways, proliferation and regulates metabolic processes (Varfolomeev and Vucic 2018). While IL-1β, an inflammatory signal trigger a synthesis and accumulation of pro- IL-1β stored in cytosolic which will activate a formation of inflammasome leading to CASP1 activation and pro- IL-1β processing, and a secretion of active IL-1β. The active IL-1β has a role in stimulating immunity cells such as macrophage, B-cell and T-cell differentiation and also induce prostaglandin production.

In this study, mRNA of TNF- α exhibited the highest level in both control and stimulated cells when compared to other target genes. TNF- α increased but not significantly after PGN stimulation suggesting that the amount of mature TNF-α, which was processed from the TNF-α precursor presented on the cell membrane, was sufficient to regulate the inflammatory system, therefore, it resulted in a small amount of TNF-α production after stimulation. In contrast, it has been reported that PGN obtained from *E.coli* could not induce the secretion of TNF-α in a human monocytes cell line (MM6) (Asong et al 2009) in this case, suggested that the increase of TNF-α mRNA might not for inflammatory purpose but for another cell functions. The similar trend showed in IL-1β as both of the cytokines (TNF- α and IL-1β) have a similar pattern of production and display overlapping function.

In terms of IL-6, this cytokine has a function in both pro-and anti-inflammatory, and be a major player in hematopoiesis, B cells differentiation and cytotoxic T cell activation (Duque and Descoteaux 2014).

IL-6 promotes the production of acute-phase protein and induce fever as pro-inflammatory cytokine when it activated by TNF- α and IL-1β. This cytokine is synthesized in ER but is not secreted as a precursor like TNF-α and IL-1β. Therefore, there are a small amount of IL-6 available in the macrophages during normal condition. This is similar to the result of IL-6 activation which presented significantly upregulation of IL-6 mRNA after PGN activation which was approximately 43 times higher than it amount of mRNA in control. The pro-inflammatory cytokines response to PAMPs has been reported in many studies which were provided in Table 3.8.

For anti-inflammatory cytokines (IL-10 and TGF-β), have an antagonistic function to pro-inflammatory cytokines, as they have a role in suppression pro-inflammatory secretion to reduce inflammation and promote a healing stage. TGF-β is a regulatory cytokine which involved in cell proliferation, differentiation, migration and apoptosis under physiological and pathological conditions and TGF-β also limit the proliferation of T cell by inhibiting the production of IL-2 (Li et al 2006; Yang and Zhou 2008). Whereas, IL-10 is a key role in inhibition of cytokines synthesis which is mainly exerted against TNF-α, IL-1β, IL-6, GM-CSF and IFN-γ (reviewed in Fioranelli and Roccia 2014) and also directly inhibit reactive radical species, phagocytosis and antigen presenting process which indirectly limit function of Th cells (Piazzon et al 2015). It is known that production of IL-10 in monocytes is secreted shortly after production of pro-inflammatory cytokines such as TNFa, IL-1, IL-6 and IL-8 (Zou et al 2003) which was similar to the result of this study. The result revealed down-regulation of IL-10 and TGF-β mRNA at 6 hours post-activation and then up-regulation after 12 hours post-activation suggest a bi-directional control between pro-inflammatory cytokines and anti-inflammatory cytokines and it have been reported in a study of the interaction between TGF-β and IL-1β in head kidney leukocytes of grass carp (Yang et al 2014). Moreover, it has been reported that TNF-α in human peripheral blood mononuclear cell was able to enhance the production of IL-10 (Kube et al 1995) and a similar result has been reported in pufferfish (Zou et al 2003).

In general, it is necessary to balance an expression between pro and anti-inflammatory cytokines in order to maintain a proper function of the immune response, particularly IL-10 which has multiple roles in the immune system. Lack of IL-10 resulted in hypersensitive reaction, while overexpression of IL-10 is increased a change of tumor or cancer development and Th2 dependent autoimmune (Fioranelli and Roccia 2014). Moreover, the up-regulation of IL-10 leads to an improper pathogen elimination of host and involved in the survival of intracellular pathogen such as *Leishmania donovani*,

Mycobacterium tuberculosis, Trypanosoma cruzi and *Coxiella burnetii* and It is stimulated by various mechanisms of the pathogen for creating a suitable intracellular environment for pathogen survival (reviewed in Verma et al 2016) which contribute a chronic stage of infection. Similarly to the result of this study, the amount of IL-10 mRNA was upregulated approximately 4.5 times at 12 hours after stimulated with PGN, revealed an imbalance between pro and anti-inflammatory cytokines. It was suggested a chronic stage of the cell which might be an effect of culture condition or contamination of intracellular pathogen.

3.4.3 Response of innate immune system to viral infection

Antiviral innate immunity pathway is the first line of defense against an infectious virus by secretion of type I interferons (IFN) which exhibit antiviral, anti-proliferative and immunomodulatory functions (Honda et al 2005; Seth et al 2006). Upon infection target cell, Toll-like receptors (TLRs) on surface or endosome, which are a major PRR of a host, recognize PAMP of virus and trigger a signalling cascade resulted in transcription of antiviral factors. Expression of TLRs is mainly expressed in antigen presenting cells (APCs) including macrophages, dendritic cell and B-cells (Mogensen 2009). TLR2 mainly recognizes bacterial production resides at the cell surface, while TLR3, TLR8 and TLR9 are expressed in the intracellular compartment; endosomes and lysosomes. TLR3 recognizes dsRNA virus and dsRNA release from a death cell. TLR8 is sensitive to G/U-rich ssRNA virus in endosome and TLR9 is a receptor for DNA virus by recognizing unmethylated CpG DNA (Seth et al 2006). Downstream signalling genes of antiviral response after activated TLRs which were used in this study were Interferon regulatory factor3 (IRF3), Viperin or RSAD2 (radical SAM domain-containing2) and IL-10. IRF3 is a transcription factor that regulates transcription of IFN gene by producing a protein complex called enhanceosome which consists of several transcription factors such as ATF-2/c-Jun, NF-κB and IRF3. The enhanceosome is binding at a promotor of IFN for the gene transcription (Maniatis et al 1998; Seth et al 2006). Whereas, Viperin is an antiviral protein which is strongly induced by IFNs, virus, LPS and poly I:C.

Results of this study revealed an upregulation of TLR3 mRNA while that of other TLRs were down-regulated. The downstream signalling genes expression; IRF3 and viperin showed a significant upregulation. However, the upregulation of viperin might be induced by poly I:C or IFN, as viperin is able to activate IRF3 to produce IFNs which act as an inducer of viperin gene. For IL-10, the mRNA of IL-10 increased upon stimulation with poly I:C. This result is in disagreement with a general function of

this gene, as IL-10 is known as an anti-inflammatory cytokine which is suppressed by type I IFNs (Feng et al 2002). However, the result was coherent with the expression of IL-10 after stimulation with PGN, it is suggested an abnormal function of IL-10 in Nile tilapia which require further study for better clarification. The response of innate immunity genes to various types of PAMPs in teleosts have been reported in serveral study, some of the studies are provided in Table 3.6.

Table 3.6 Example of innate immunity genes response to various PAMPs

Genes	organism	Finding	Ref.
TNF-α	Common carp	head kidney; induced upon LPS stimulation	(Saeij et al 2003)
	Rainbow trout	macrophages; up-regulated after stimulation with LPS and poly(I:C)	(Hong et al 2013)
	Zebrafish	head kidney; increased upon LPS and poly(I:C) injection, the highest level of expression presented at 3 h after injection then gradually decreased	(Varela et al 2012)
IL-1β	European eel	macrophages; up-regulated after stimulation with PGN	(Callol et al 2013)
	Atlantic cod	head kidney; up-regulated upon LPS treatment but not response to poly(I:C)	(Seppola et al 2008)
	Japanese flounder	head kidney and spleen; up-regulated upon LPS treatment	(Taechavasonyoo et al 2013)
	Salmonids	RTS-11 and head kidney macrophage; increased after LPS and poly(I:C) stimulation	(Husain et al 2012)
	Zebrafish	head kidney; increased upon LPS and poly(I:C) injection, the highest level of expression presented at 3 h after injection then gradually decreased	(Varela et al 2012)
IL-6	Large yellow croaker	head kidney; increased upon LPS injection	(Zhu, Q. et al 2016)
	Zebrafish	head kidney; increased upon LPS and poly(I:C) injection, the highest level of expression presented at 3 h after injection then gradually decreased	(Varela et al 2012)

	Rainbow trout	macrophage; can be induced by LPS, poly I:C, and IL-1	(Costa et al 2011)
IL-10	Atlantic cod	spleen; highly up-regulation after i.p. injection with poly(I:C)	(Seppola et al 2008)
	Goldfish	granulocytes and monocytes; reduced after stimulation with rTNF-α	(Grayfer et al 2011)
	Zebrafish	kidney, gill and gut; upregulated after LPS stimulation	(Zhang et al 2005)
	Common carp	head kidney and liver; expressed after LPS stimulation	(Savan et al 2003)
	Indian major carp	gill, liver and kidney; increased following *A. hydrophila* injection	(Swain et al 2011)
TGF-β	Goldfish	macrophages; up-regulated after treated with LPS or rTNF-a	(Haddad et al 2008)
	Nile tilapia	head kidney and spleen leukocytes; up-regulated upon LPS or poly(I:C) treatment	(Zhan et al 2015)
	Grass carp	head kidney leukocytes; up-regulated upon LPS stimulation	(Wang, X. et al 2016)
IRF3	Nile tilapia	various tissues; increased upon poly(I:C) treatment	(Gu et al 2016)
	Miiuy croaker	head kidney macrophages; up-regulated upon poly(I:C) treatment	(Shu et al 2016)
	Japanese flounder	head kidney and gill; up-regulated upon poly(I:C) injection	(Hu et al 2011)
Viperin	Nile tilapia	kidney, liver, spleen, and gills; upregulated upon induction with LPS and poly(I:C)	(Lee et al 2013)
	Large yellow croaker	blood, head kidney, spleen and liver; upregulation upon poly(I:C) stimulation	(Zhang, Jianshe et al 2018)
	Mandarin fish	gill; up-regulated upon infection of virus and poly(I:C)	(Sun and Nie 2004)
TLR2	European eel	macrophages; down-regulated after stimulation with PGN	(Callol et al 2013)
	Turbot	tissues; up-regulated upon *V. anguillarum* and *S. iniae* infection	(Liu et al 2016)

	Gibel carp	spleen; up-regulated after i.p. injection with CyHV-2	(Fan et al 2018)
TLR3	Large yellow-croaker	spleen, blood and liver; induced by poly(I:C) injection	(Huang, X. et al 2011)
	Orange-spotted grouper	liver; up-regulated after poly(I:C) challenge	(Lin, K. et al 2013)
	Gibel carp	head kidney; up-regulated after i.p. injection with CyHV-2	(Fan et al 2018)
	Sea perch	head kidney, spleen and liver; up-regulated after *V. harveyi* and *S.agalactiae* infection	(Wang, P. et al 2018)
TLR8	Rainbow trout	anterior kidney leukocytes were not affected by poly I:C or R848 treatments	(Palti et al 2010)
	Turbot	intestine, skin and gill; induced upon *V. anguillarum* and *S. iniae* infection	(Dong, X. et al 2016)
	Large yellow-croaker	head kidney and spleen; up-regulated upon poly I:C treatment	(Qian et al 2013)
	Golden pompano	head kidney and spleen; up-regulated upon poly I:C treatment but not response to *V. alginilyticus* infection	(Wei, Y. et al 2017)
TLR9	Turbot	intestine, skin and gill; induced upon *V. anguillarum* and *S. iniae* infection	(Dong, X. et al 2016)
	Golden pompano	head kidney and spleen; up-regulated upon *V. alginilyticus* infection but not response to poly I:C treatment	(Wei, Y. et al 2017)

3.5 Conclusions

This study shows that the macrophage primary cell culture derived from the head kidney of Nile tilapia is an effective model to study the interaction of host PRRs upon exposure to PAMPs which will facilitate further study of the immune response in the Nile tilapia. Besides, it suggests the importance of innate immune system in teleost, as they are living under high pathogenic pressure, thus a rapid response to invading pathogens is necessary. Moreover, according to the phylogenetic analysis, the innate immunity genes analysed showed high sequence similarity across different orders of taxa or diverse living behavior. Therefore, results indicate a conservation of PRRs during evolutionary events. However, there are some results that need further examination such as the elevated expression of the target genes analysed in macrophages from naïve fish.

Chapter 4

Mycobacterium spp. detection in Nile tilapia (*Oreochromis niloticus*)

4.1 Introduction

Mycobacterium spp. are rod-shaped, Gram-positive, acid-fast, non-motile and non-spore forming bacteria (Pourahmad et al 2014). They commonly found in an aquatic environment and are known as pathogenic to fish in marine, freshwater and brackish water. There are several species of mycobacteria that can infect fish and cause tuberculosis, including *M. fortuitum, M. flavescens, M. chelonae, M. gordonae, M. terrae, M. triviale, M. diernhoferi, M. celatum, M. kansasii ,M. intracellulare* and *M. marinum* (Puk et al 2017). A culture system tends to increase the susceptibility of the animal to the disease such as intensive culture and circulating water system particularly, laboratory fish as zebrafish. This bacteria has been reported as important zoonotic pathogen which have a wide range of host, not only aquatic animal but also amphibians, reptiles and mammalian (Francis-Floyd 2011). A potential way of Mycobacterium infection in fish include the ingestion of contaminated food, debris tissues and through gill or skin lesions where the bacteria are able to survive for year by suppression immune system and macrophages activity of host.

As Mycobacterium is an intracellular parasite, reside in a phagocytic cell of a host by suppressing microbicidal mechanisms of the host to make a suitable intra-phagosomal environment for its growth (Flynn and Chan 2003) and stimulate phagocyte to enable cytokines production of host which promote a proper condition for its survival (Falcone et al 1994). Therefore, in early stages of infection, Mycobacterium is engulfed by host phagocytic cells such as macrophages where the bacterial cell may be eliminated or be carried inside macrophages cell. An interleukin was triggered to recruit macrophages, dendritic cells, lymphocytes and epithelioid cells of the host to form a granuloma which prevent further spread of the bacteria to surrounding tissue. In general, Mycobacterium can stay arrested in the granuloma for a long time. However, immunosuppression can breakdown the balance

between host control and pathogen, resulting in bacterial growth and progression of the infection (reviewed in Fell et al 2016).

Mycobacteriosis or fish tuberculosis external clinical signs, which are nonspecific, include scale loss, dermal ulceration, pigmentary changes and abnormal behavior, whereas internal clinical signs include enlargement of the spleen, kidney or liver and grey or white nodules in internal organs. However, if the infection is chronic, fish might not show any clinical signs (Lara-Flores et al., 2014). A pathohistological examination of Mycobacterium revealed that early granulomas consist of group of macrophages in the central area surrounded by lymphocytes and plasma cells whereas late granulomas have a tightly packed epithelial cells as an outer layer covering a group of macrophages, granulocytes and lymphocytes in a central area where a presence of acid-fast mycobacteria (Puk et al 2017). Detection of Mycobacterium traditionally relies on histological examination and bacteriology studies to confirm the presence of granulomatous lesions and estimate the number of bacteria to determine the severity of the infection. However, in some circumstances granuloma detection can lead to false positive results because other pathogens such as parasites can also cause granuloma and clinical signs are difficult to observe at early stages of infection as the bacteria can stay at arrested stage in the host for a long time before appearance the disease (subclinical infection) (Pourahmad et al 2014).

According to the results on the macrophage primary cell cultures (Chapter 2) and their response to pathogen infection (Chapter 3), it was noted that the characteristics and behaviour of the macrophages derived from head kidney of control/healthy Nile tilapia were always de-attached from the culture surface, formed granulomas after 3 days of culture and had short life span under the culture conditions (Fig. 3.9). These observations were considered as unusual especially when compared to macrophage primary cell cultures of other fish such as trout or salmon. Moreover, macrophages from control/unstimulated groups showed high levels of mRNA for a number of the immune-related studied genes (Fig. 3.10). These results suggested that the cells were already on an activated stage which could be due to environmental stress or pathogen infection. However, the sampled fish were apparently healthy and showed no clinical sign of infection. As Mycobacterium has been shown to establish in cultured fish without progressing to disease this aimed to detect the presence of Mycobacterium in Nile tilapia held at the Tropical Aquarium (Institute of Aquaculture, University of Stirling) by using PCR-based method to increase efficiency and sensitivity of Mycobacterium detection.

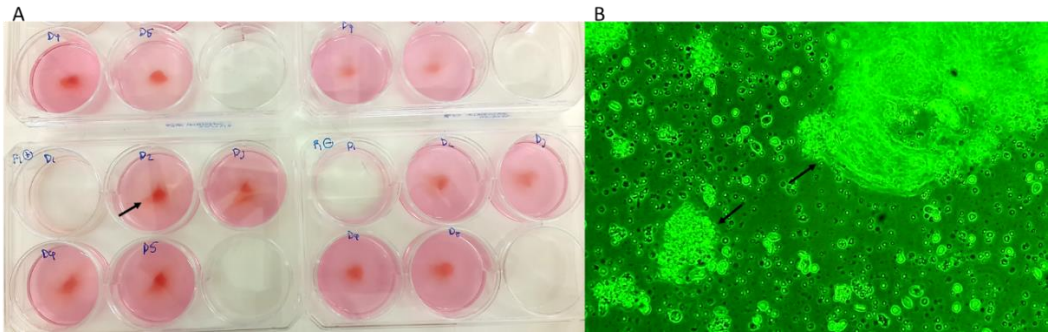


Figure 4.1 Macrophage primary cell culture of Nile tilapia. A: a red cloud of the cell after seeding to cell culture plate B: the cell granulomas on 4 days of culture, the cell were observed under 20X objective

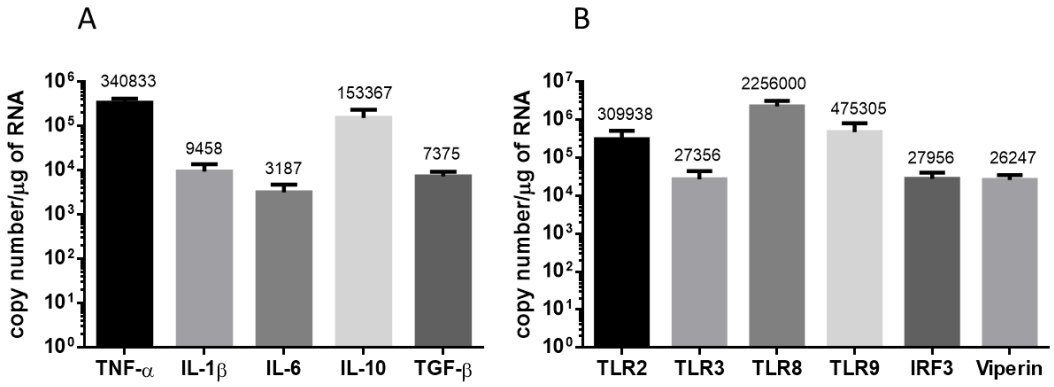


Figure 4.2 The mRNA copy number of innate immunity genes of control cell

4.2 Material and Methods

4.2.1 *Mycobacterium* detection

4.2.1.1 Samples collection

An infection of Mycobacterium in Nile tilapia was investigated in both tissue samples and macrophage cells. Head kidney, spleen, gill, muscle and mucus of Nile tilapia (n=1) (198 g) were collected to detect Mycobacterium in tissues. On the other hand, head kidney of 3 Nile tilapia of an average weight of 200.5±5.3 g.were collected for macropahges primary cell culture. Sampled Nile tilapias were apperantly healthy and showed no skin lesion or clinical sings of mycobacteriosis . Besides, sampled fish were sourced from the same tank. Samples were stored at -70 °C for DNA extraction. Macrophage primary cells derived from the head kidney of Nile tilapia were cultured in condition as previously described in 2.2.1.2. Cells were incubated at 28 °C with 3 % CO_2 and harvested every day until 5 days for DNA extraction. DNA extraction of tissue samples and macrophages wwas performed with DNeasy Blood & Tissue Kits (Qiagen, MD, USA). DNA quality was measured with Nanodrop® ND-1000 (ThermoScientific, MA, USA) before stored at -20 °C until use.

4.2.1.2 Primers design and Nested PCR

DNA samples of Nile tilapia were amplified with genus-specific primers of Mycobacterium which were designed from a highly conserved region of 16S rRNA (Talaat et al 1997) using an automated thermal cycler (Biometra®, Jena, Germany). The PCR product was measured by 1.5% Agarose gel electrophoresis and visualized under the UV light. A PCR band, which presented 924 bp, was purified with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Duren, Germany) for sequencing. The sequence was blasted using Blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for Mycobacterium nucleotides conformation. Then, the DNA sequence obtained from the genus-specific primers was used as a template to design primers for a nested PCR. The nested PCR primers were designed using a method described in 3.2.1.1. In order to perform a nested PCR, this method requires 2 pairs of primers and 2 runs of PCR, therefore the genus-specific primers were used as primary primers, and the nested primers were used as secondary primers. The PCR product of the first PCR was diluted to 10-2 and used

as a template for the second PCR. PCR mixture and condition was described in Table 4.1. Moreover, the species of Mycobacterium was identified using species-specific primers for *M. Marinum*, *M. Haemophilum* and *M. chelonae* which were designed from HSP65 gene (Meritet et al 2017). The sequences of primers and annealing temperature used in this experiment were described in Table 4.2.

Table 4.1 Nested PCR protocol

1st PCR Component	Reaction
Template (DNA)	2 µl
10 mM Fowords 1st primer	1 µl
10 mM Reverse 1st primer	1 µl
PCR mix (mytaq)	10 µl
DNAse/RNAse free water	6 µl
Final volume	20 µl

2nd PCR Component	Reaction
1st PCR product	2 µl
10 mM Fowords 2ndprimer	1 µl
10 mM Reverse 2nd primer	1 µl
PCR mix (mytaq)	10 µl
DNAse/RNAse free water	6 µl
Final volume	20 µl

Step	Temperature	Time	
Pre-denaturation	95 °C	1 min	
Denaturation	95 °C	15 sec	30 cycles
Annealing	61 °C	10 sec	
Extension	72 °C	10 sec	
Final extension	72 °C	1 min	

Table 4.2 List of Mycobacterium primers with product size, annealing temperature and citation

Genes	Sequences	Product size	T_a	Reference
Genus-specific primers				
Mycobacterium	>F GCGAACGGGTGAGTAACACG	924 bp	61 ˚C	(Talaat et al 1997)
	>R TGCACACAGGCCACAAGGGA			
Species-specific primers				
M. marinum	>F CAACCC GCTCGGTCTGAA	84 bp	59 ˚C	(Meritet et al 2017)
	>R CGACCTCTTTGGCCGACT T			
M. chelonae	>F AAGGAAGTTGCCAAGAAGACTGA	75 bp	58 ˚C	(Meritet et al 2017)
	>R CAGAGCCTGGGCAAGCA			
M. haemophilum	>F GTTAAGGTGGCGTTGGAAGCT	56 bp	58 ˚C	(Meritet et al 2017)
	>R TCCAGCCCGGAGTTGAAG			

4.3 Results

In order to detect Mycobacterium in Nile tilapia, head kidney spleen, gill, muscle and mucus of Nile tilapia (n=1) were collected and the DNA was extracted for amplification with genus-specific primers of *Mycobacterium* (Talaat et al 1997). Moreover, macrophage primary cell derived from head kidney (n=3) were cultured and harvested every day for 5 days to detect Mycobacterium in macrophage cells. Using genus-specific primers, it was possible to detect *Mycobacterium* DNA in spleen, gill, muscle, mucus and macrophages primary cell cultures derived from head kidney but could not detect *Mycobacterium* DNA in head kidney tissue of Nile tilapia (Figure 4.4A and 4.4B). An agarose gel electrophoresis appeared a single band of PCR product (924 bp) in macrophage but no band appeared in tissues. However, it required a high amount of DNA template which was 400 ng per sample. Therefore, a nested PCR is required to increase the sensitivity of PCR for Mycobacterium detection and reduction of the amount of DNA template.

Nested PCR primers were designed from a sequence of the genus-specific amplicon. Product size of nested PCR was 172 bp and the amount of DNA template was 50 ng/reaction. It is found that there was *Mycobacterium* detection in spleen, gill, mouth and muscle of Nile tilapia but could not be detected in the head kidney. Whereas, in macrophages, the band of PCR products were more obvious than using only genus-specific primers. Moreover, the result highlight that the mycrobacterium DNA was able to detect on macrophage primary cell culture derived from head kideney but could not detect theb DNA in head kidney tissue. As a result, the presence of strong bands of PCR product after nested PCR revealed an infection of Mycobacterium in Nile tilapia samples. In the next step, a species of Mycobacterium was identified using species-specific primers (Meritet et al 2017); *M. marinum, M. chelonae* and *M. haemophilum*. The result showed that there were no primer specific bands present in tissue samples or macrophages Nile tilapia samples (Fig 4.5). It could be implied that the mycobacterium which infected Nile tilapia samples was none of these 3 species, and required further study to identify the species of this Mycobactereium.

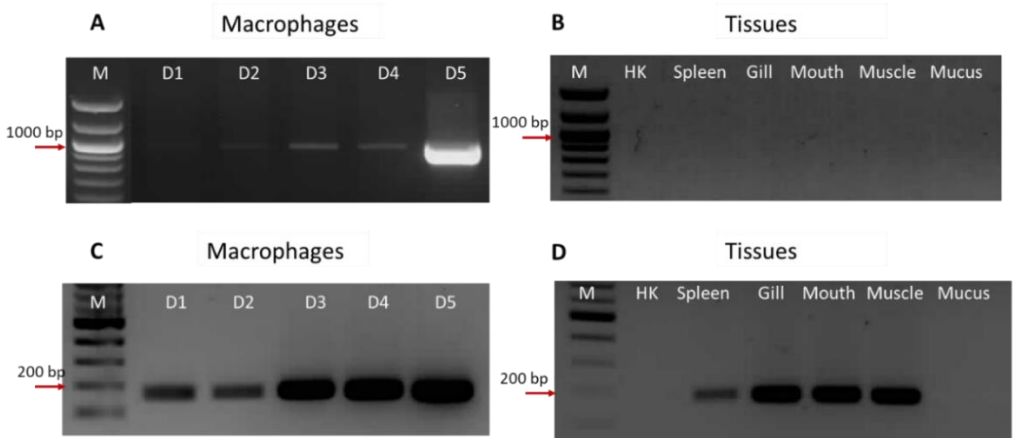


Figure 4.3 Agarose gel electrophoresis images of mycobacterium detection in Nile tilapia. A: a detection of mycobacterium in macrophage from day 1-day 5 using genus-specific primers and 400 ng of DNA template, B: a detection of mycobacterium in tissues using genus-specific primers and 400 ng of DNA template, C: nested PCR to detect mycobacterium in macrophage from day 1-day 5 using 50 ng of DNA template, D: nested PCR to detect mycobacterium in tissues using 50 ng of DNA template. A product size of genus specific primers; 924 bp and a product of nested primers; 172 bp.

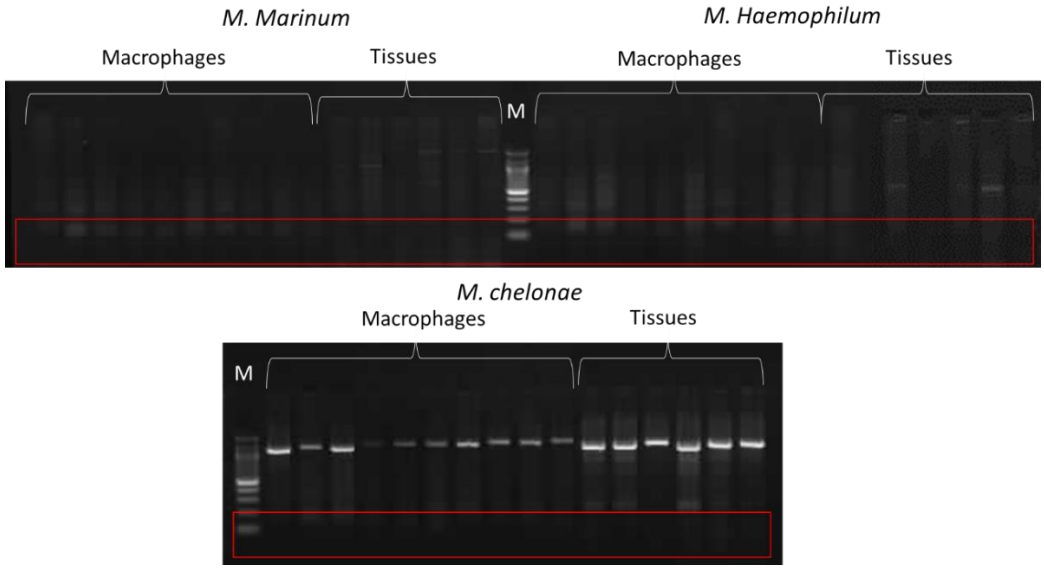


Figure 4.4 Agarose gel electrophoresis images of mycobacterium species detection in macrophages and tissues of Nile tilapia. A red box is an expected size of PCR product; *M.marinum* (84 bp), *M. hasemophilum* (75bp) and *M.clelonae* (56bp).

4.4 Discussion

Mycobacterium detection has traditionally relied on histological and bacteriology methods to confirm the existance of granulomatous lesions and estimate the number of bacteria to determine the severity of the infection. However, in some circumstances granuloma detection can lead to false positive result as other pathogens also can cause granulomas such as parasites, and it is difficult to detect early stages of infection (Pourahmad et al 2014). As a result, histological and bacteriology methods have low specificity and sensitivity in comparison with molecular methods that allow the detection of bacteria at a very beginning or low level of infection. It has been reported that the PCR was possible to detect a very low amount of *Mycobacterium* DNA at 10 fg or approximately 10 mycobacteria in goldfish, which was 1,000 times more sensitive than histological study (Pourahmad et al 2014).

Therefore, in this study, a PCR based method was used to investigate the presence of *Mycobacterium* DNA and increased its sensitivity by the development of nested-PCR. The nested-PCR was developed using internal primers of the 16S rRNA primers. Moreover, a macrophage primary cell culture derived from head kidney was used to study the distribution of Mycobacterium compare with other tissues of Nile tilapia. The results indicated that *Mycobacterium* primarily distributed in macrophages derived from head kidney, which could be detected with normal PCR, followed by muscle tissue, gill and spleen, which could be detected with nested-PCR. However, *Mycobacterium* DNA could not detect in head kidney tissue and mucus. These results are in agreement with the nature of *Mycobacterium* which is known as intracellular parasites of phagocytic cells, therefore this bacteria was easily found in macrophages, which are major phagocytic cells of the host, followed by muscle tissue and spleen. Moreover, *Mycobacterium* DNA was detected in macrophages primary cells daily for 5 days, a low level of mycobacterium DNA was observed on an early day of culture, when the cell was attached on the surface and found some group of the cell. The bacteria then increased on a later day when granulomas and cell mortality were found. This data suggest that the macrophages can control the growth of bacteria inside the cell, then the bacteria were released and increased massively after the macrophages die. As *Mycobacterium* was arrested inside a granuloma, which is a formation of phagocytic cell of the host to prevent bacterial transmission to nearby tissue, and can be survive for a long time before showing clincal signs of infection (Fell et al 2016). The presence of Mycobacterium in macropahges was involed in a manipulation of host cytokines production, as it was crucial for intracellular survival of bacteria or elimination by antimicrobial armed of host (Grayfer et al 2011). This

nature of Mycobacterium also found this study as Nile tilapia were apparerantly healthy, no skin lesion or clinical sign of mycobateriosis.

Moreover, in this study, we have tried to identify the species of *Mycobacterium* present in Nile tilapia using molecular information from other studies . The DNA isolated from macrophages was amplified with specific primers of 3 commonly *Mycobacterium* species identified in Zebrafish (*Danio rerio*) (Meritet et al 2017); *M. marinum, M. chelonae*, and *M. haemophilum.* Results showed no specific band present after amplification. However, there were many *Mycobacterium* species which are pathogenic species in fish as presented in Table 3.9. Moreover, an outbreak of mycobacteriosis in farm is not likely caused by only one species of *Mycobacterium*. An identification of *Mycobacterium* species during the disease outbreak in Nile tilapia farm, in Mexico was able to isolate 3 species of this bacteria from liver, spleen and skin lesion; *M. marinum, M. fortuitum and M. parascrofulaceum*. However, *M. marinum* and *M. fortuitum* were suggested as principle species associate with Nile tilapia mortality in this study (Lara-Flores et al 2014). Therefore, specific primers of other species are required for the identification of Mycobacterium species which infected Nile tilapia in our aquarium for treatment and further prevention.

Table 4.3 A review of Mycobacterium spp. identification in fish. B/P: biochemical/phenotypic characterisation

Species	Hosts	Identification methods	References
M. marinum	goldfish	B/P 16s rRNA	(Pourahmad et al 2014)
	zebrafish	B/P *Hsp65*	(Meritet et al 2017)
	Nile tilapia	B/P 16s rRNA	(Lara-Flores et al 2014)
	Dwarf gourami	B/P MALDI-TOF, 16s rRNA, *Hsp65*	(Puk et al 2017)
M. chelonae	zebrafish	B/P *Hsp65*	(Meritet et al 2017)
M. haemophilum	zebrafish	B/P *Hsp65*	(Meritet et al 2017)
M. fortuitum	Nile tilapia	B/P 16s rRNA	(Lara-Flores et al 2014)
	Neon tetra	B/P MALDI-TOF, 16s rRNA, *Hsp65*	(Puk et al 2017)
M. peregrinum	Electric yellow cichlid	B/P MALDI-TOF, 16s rRNA, *Hsp65*	(Puk et al 2017)
M. abscesus	Ram cichlid	B/P MALDI-TOF, 16s rRNA, *Hsp65*	(Puk et al 2017)
	Goldfish	16s-23s rRNA	(Shukla et al 2018)
M. senegalense	Goldfish	16s-23s rRNA	(Shukla et al 2018)
M. parascrofulaceum	Dwarf gourami	16s-23s rRNA	(Shukla et al 2018)

4.5 Conclusions

Results of this study suggest a high efficiency and sensitivity of nested PCR method to detect a low level of *Mycobacterium* DNA present in tissues and macrophage cells of Nile tilapia which is useful tool to identifiy *Mycobacterium* at an early stage of infection or low level of bacteria. Moreover, the use of macropahges primary cell culture derived from head kidney presented a higher possibility in *Mycobacterium* detection than tissue samples and it has ability to use as an alterantive approch in *Mycobacterium* isolation and culture. Besides, the result of this study is a comformation of *Mycobacterium* contamination in Nile tilapia culture system, in the tropical aquarium.

Chapter 5

Effect of genetic and environmental background to tilapia immunity performance

5.1 Introduction

Tilapia is known as an important commercial fish, however this term has been used as common name for more than 70 species of "Cichlid" fish. Of these, nine species; *O. niloticus, O. aureus, O. mossambicus, O. spilurus, O. hornorum, Sarotherodon galilaeus, S.melanotheron, Tilapia rendalii, T. zillii.* However, only Nile tilapia (*O. niloticus*), Mozambique tilapia (*O. mossambicus*) and Blue tilapia (*O. aureus*) are important aquaculture species worldwide, particularly Nile tilapia which is a major aquaculture species. In 2017, Nile tilapia production accounted to 69 percent of global Tilapia production (FAO,2019). Historically, Mozambique tilapia was the first species which was introduced from Africa to Asian countries for culture, due to its undesirable characteristics and small production value it success was only partial. Even though, Mozambique tilapia has high fecundity and high saline tolerance (up to 20 ppt), but it has early maturation and poor aquaculture potential. Therefore, it is commonly used for hybridization (Gupta, 2014) such as production of red hybrid tilapia strain. Similarly, Blue tilapia which is suitable to produce monosex tilapia and cold tolerance strain as it can tolerant to low temperature to 8-9 ˚C has been used for hybridization purposes (Beveridge and McAndrew 2012). On the other hand Nile tilapia became popular in the latter haft of 20Th century, as it presented a better performance over the other strains in a variety of aquaculture environments, it is fast growing and easily to reproduce. Moreover, the success in GIFT project which managed to reduce unwanted traits and increase desirable traits such as increased growth brought Nile tilapia to be the dominant species of Tilapia in aquaculture.

In Thailand, tilapia culture plays an important role in aquaculture business as its production accounted to 52 % of overall freshwater fish production in 2017 and 95 % of the product was consumed within country (DOF ,2019). The total tilapia production was a conclusion of both Nile tilapia and red tilapia

production. The cultivation of tilapia in Thailand was initiated approximately 60 years ago when Nile tilapia was introduced in 1966. Since then, several strains of Nile tilapia were developed to meet both farmers and market needs, however they basically originated from Chitralada strain. According to Pongthana (2010), there are 4 popular strains of Nile tilapia in Thailand (Figure 5.1);

i) Chitralada1 strain; it was developed using within family selection for 5 generations before distributed to farmers by the department of fishery in 1993.

ii) Chitralada2 strain; it was released in 1995, this strain is a Genetically Male Tilapia (GMT). It was crossed between Supermale (YY male) and normal Nile tilapia female. The production yield of this strain compared with normal male tilapia is higher and has similarity in a size of the fish in the same pond.

iii) GIFT strain; it was developed by FAO and partners in the Philippines in 1988 and used Chitralada as one of base population for selective breeding. The 5^{Th} and 9^{th} generation of the GIFT was imported to Thailand for further study. The final generation of GIFT strain showed a better growth rate than normal tilapia approximately 85 %.

iv) Chitralada3 strain; it is result of further development of the 5^{th} generation of GIFT strain using mass selection. After 3 generations of selection, Chitralada3 has 40 % better growth rate than the first generation.

In terms of Red tilapia, there are 6 strains of Red tilapia that has been reported by Pongthana (2010) (Figure 5.1)

i) Thai red tilapia strain; the hybrid between male Mozambique tilapia and female Nile tilapia which has red to white with some dark spot of body colour and tolerates salinity up to 17 ppt.

ii) Pathumthani red tilapia strain; which was developed from a cross breeding program of 4 red tilapia strains from Thailand, Taiwan, Stirling and Malaysia. This strain has pink to orange body colour, fast growing, high production yield and tolerate salinity to 25-30 ppt. which is suitable for culture in brackish water and polyculture in shrimp pond.

iii) CP red tilapia strain; which was developed by the CP company, the body colour of this strain is red to orange.

iv) Taiwanese red tilapia strain; a cross breeding between red mutant of male Mozambique tilapia and female Nile tilapia. This strain is extremely salinity tolerant up to 34 ppt. and can be cultured in sea water.

v) Malaysian red tilapia strain; a cross breeding of male Mozambique tilapia and female Nile tilapia developed by Fishery research institute of Malaysia.

vi) Stirling red tilapia strain; this strain was developed by the Institute of Aquaculture, University of Stirling by breeding between a recessive red colour of Nile tilapia.

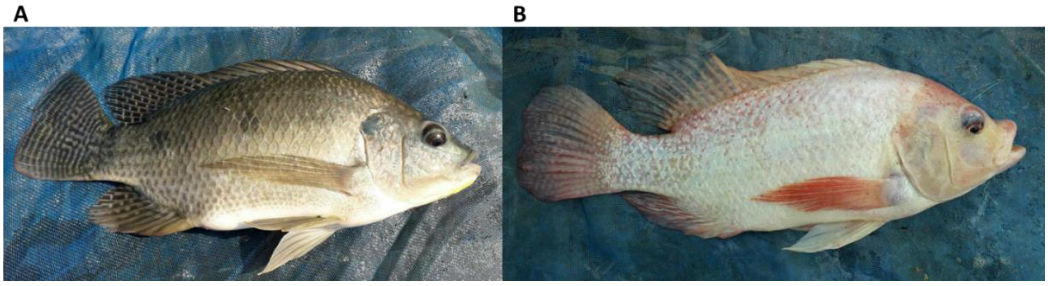


Figure 5.1 Strain of tilapia in Thailand, A: Nile tilapia (*Oreochromis niloticus*) (Chitralada3) and B: Red tilapia (*O. mossambicus X O. niloticus*) (Ritchuay 2018)

As there are variety of tilapia strains available in Thailand, strain selection for cultivation depends on farming environment and market preference. However, the most famous strain of Nile tilapia is Chitralada3. This strain is produced by the Department of Fishery which distributes its fry and fingerling to farmers for free as part of agriculture extension campaign to support household economy and smallholder farm. Whereas, red tilapia, both Thai red tilapia strain and Pathumthani red tilapia strain are also part of the agriculture extension campaign but the data of the most popular cultured strain is not clear due to a rapid release of new red tilapia strain from private hatchery companies. Besides, red tilapia has higher market price than Nile tilapia due to its appearance and meat quality is more preferable to consumers than Nile tilapia. However, there is no specific comparison data between a total production Nile tilapia and red tilapia available.

In terms of culture environment, tilapia can be culture in monoculture or polyculture systems, in the various environment; earth pond, floating cage, paddy field or shrimp pond (Fig 5.2). However, there are some limitation of Nile tilapia and red hybrid tilapia culture such as Nile tilapia is generally cultured

in earth pond, paddy field but not favored to culture in shrimp farm due to it lower tolerance to brackish water compared to red hybrid tilapia. Besides, Nile tilapia is not a preferable strain to culture in a floating cages along the river as floating cages requires high cost during the production but the market price is low. Due to floating cage in the river, is a monoculture system, the fish rely only on commercial feed unlike polyculture or co-culture system in pond or paddy field where natural feed is available. Whereas, red hybrid tilapia is mostly cultured in the floating cages along the river and earth ponds but not recommend to culture in paddy field due to its bright colour which is difficult to protect from a predator in shallow waters (Pers.Comm.,2018).

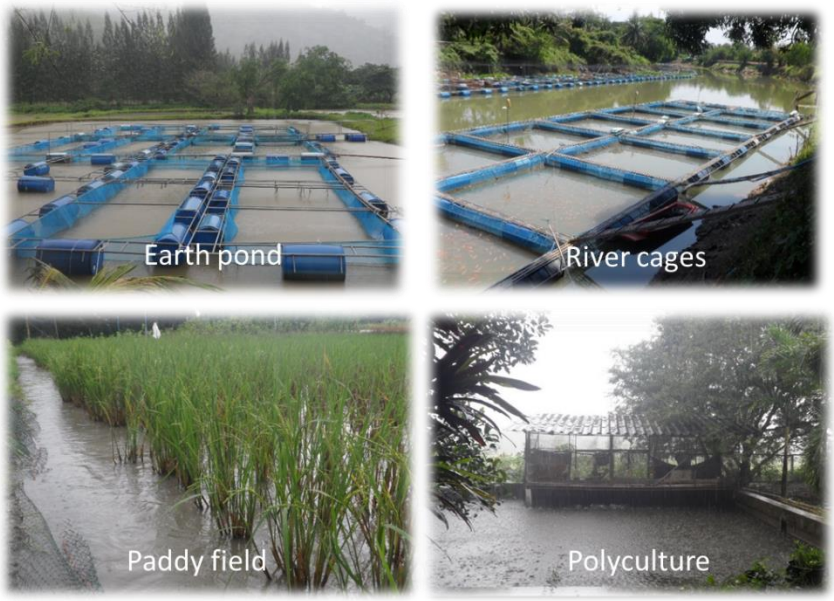


Figure 5.2 Example of tilapia culture conditions in Thailand Top left: cage culture in earth pond, Top right: cage culture in the river, Bottom right: Nile tilapia culture inthe paddy field, Bottom right: polyculture in the pond (Ritchuay 2018)

Due increased demand of tilapia has resulted in an increase in intensive production systems, which can impact environmental conditions that leads to disease and poor fish health. This is not only because of high stocking densities but also because of poor environmental conditions per se that influences fish stress and triggers its immune system. Particularly water quality is key to fish immunity and production, it was found that temperature and DO between 27-30°C and 5-23 mg/l can increase the growth rate of tilapia but the growth rate reduced when conductivity, pH and ammonia in water increase (Makori et al 2017). Besides of culture environment, there are many factors that can influence the immune system of fish such as genetics, nutrition and pathogens. Therefore, this study aimed to

characterize the baseline transcription profiles of selected genes involved in immune response among different tissues of healthy Tilapia in various culture conditiosn in order to examine the interaction between the culture environment and the strain of tilapia for supporting Tilapia fish farm management in the future.

5.2 Material and Methods

5.2.1 Tissue distribution of targeted immune system-related gene expression

Nile tilapia (*Oreochromis niloticus*) (n=9) and Mozambique tilapia (*Oreochromis mossambicus*) (n=9) (Fig 5.3) were cultured in the tropical aquarium (Institute of Aquaculture, University of Stirling) under control conditions. Fish were kept at 28 °C under 12/12 hours of light/dark photoperiodk and fed daily with a commercial diet. Fish were anaesthetized with an overdose of benzocaine before dissection. Head kidney, spleen and liver of Nile tilapia and Mozambique tilapia were collected and stored in 1 ml of TRI reagent for RNA extraction. The experiment was approved by the Animal Welfare and Ethical Review Body (AWERB) of the Institute of Aquaculture, University of Stirling, UK.

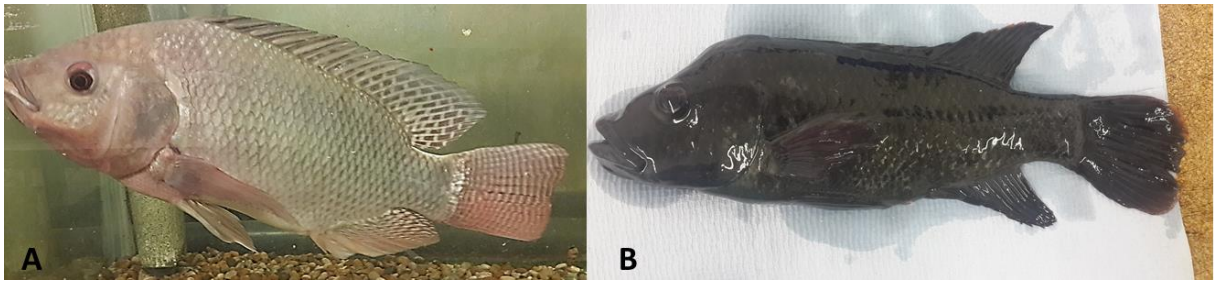


Figure 5.3 Tilapia samples in the tropical aquarium (Institute of Aquaculture, University of Stirling), A: Nile tilapia (*Oreochromis niloticus*) and B: Mozambique tilapia (*Oreochromis mossambicus*)

5.2.1.1 RNA extraction

In brief, tissue samples and TRI reagent were homogenized for 1 min or until completely homogenous. Then 100 µl of 1-bromo-3-chloropropane was added and vortexed until the colour of the mixture was milky and then incubated for 15 min at room temperature before centrifugation at 13,000 x *g* for 15

min at 4 °C. The top aqueous phase was transferred to a new tube and mixed with an equal volume of isopropanol, then allowed to stand at the room temperature for 1 h to precipitate the RNA. The mixture was centrifuged at 13,000 x *g* for 15 min at 4 °C and the liquid component discarded. The RNA pellet was washed with 1 ml of 75 % ethanol, incubated at room temperature for 5 min before centrifugation at 13,000 x *g* for 5 min, the ethanol was discarded and the resulting pellet left to dry. Finally, an appropriate volume of DNAse/RNase free water was added for dissolution and stored at -70 °C. The RNA quality was measured with a Nanodrop® ND-1000 (ThermoScientific, MA, USA).

5.2.2.2 RTqPCR

The cDNA was synthesized using the Precision nanoScript2 Reverse transcription kit (Primer Design, Chandlers Ford, UK) at a concentration of RNA/reaction of 1,500 ng. After that, the cDNA was diluted 7.5 times with RNAase/DNAse free water and stored at -20 °C. Before doing the qPCR, the cDNA was amplified with β-actin primers for quality evaluation. Then,the qPCR was performed in a 20 µl reaction with 3 µl of cDNA, 1 µl of 10 mM forwards primer, 1 µl of 10 mM reverse primer, 10 µl of Luminaris Colour HiGreen qPCR master mix and 6 µl of DNAse/RNAse free water. The cycling conditions were similar to qPCR conditions performed in chapter 3 (Table 3.3). The qPCR was done in triplicate with 5 cytokine gene targets; TNF-α, IL-1β, IL-6, IL-10 and TGF-β (Table 3.4).

5.2.2.3 Statitstical analysis

ANOVA was used to analyze the copy number of measured mRNA transcripts in tissues. Multiple comparisons were performed with Holm-Sidak test and a significance level was considered at *p*-value<0.05. The calculation was conducted with GraphPad Prism version 6.0 (San Diego, CA, USA) and graphs generated from the same program.

5.2.2 Effect of genetic background and culture environment

5.2.2.1 Sample collection

Nile tilapia (*Oreochromis niloticus*) and red hybrid *tilapia* (*O. mossambicus x O. niloticus*) (Fig 4.1) were collected from various culture environments in Thailand on April 2018. Nile tilapia samples were collected from earth ponds; monoculture system (n=14) and polyculture system (n=6), and paddy fields (monoculture; n=7) from Ratchaburi Province. While red hybrid tilapia were collected from earth ponds; monoculture system (n=13) and polyculture system (n=6) from Ratchaburi Province and floating cages along Chao Phraya river in Aung thong (n=6) and Chai-Nat (n=6) (Figure 5.4). Samples from paddy field, polyculture and floating cages were collected from private farms; Fish cultured in paddy field was fed with pellet once a day and natural feed in the field, the fish cultured in polyculture was fed twice a day with pellet and by product from chicken farm, while the fish was fed 3-4 times/day in floating cage culture. Whereas the fish cultured in monoculture system was collected from Ratchaburi Inland aquaculture research and development center, Department of Fishery, the fish was fed twice with pellet feed. Moreover, monocultured Nile tilapia and red hybrid tilapia were collected from earth ponds in the same farm in order to maximize the similarity of culture environment and decrease variation. Spleens of apparently healthy fish were sampled after the fish were anaesthetized with an overdose of benzocaine and the tissues were preserved in 1 ml of RNA later for RNA extraction.

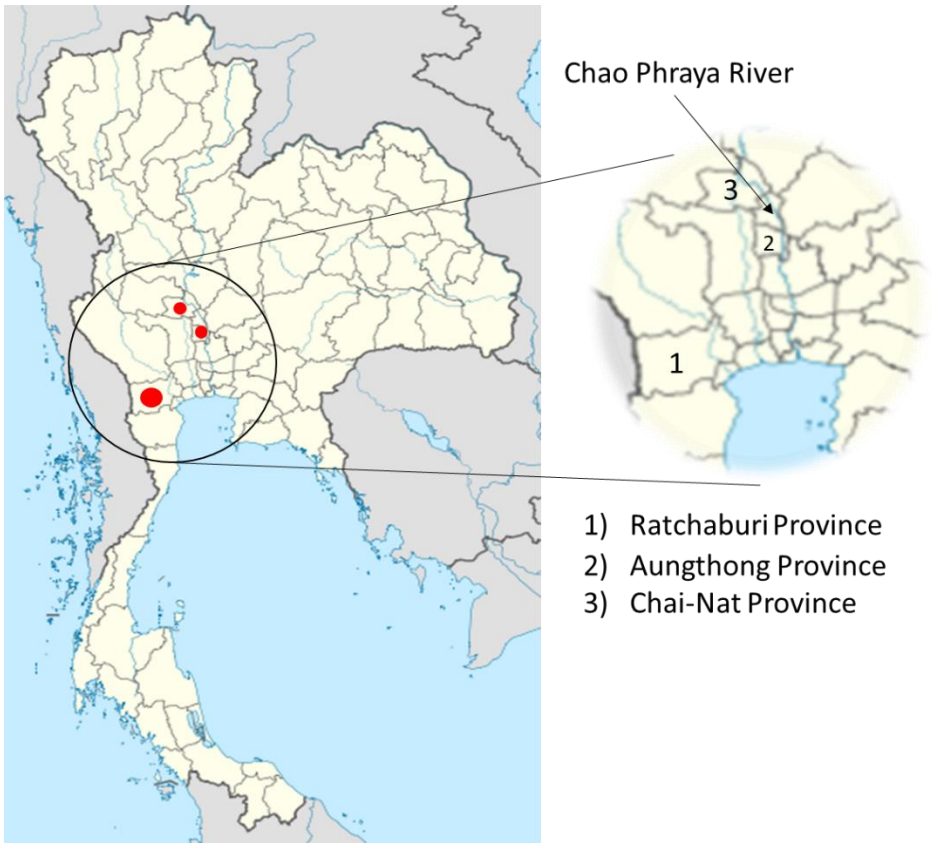


Figure 5.4 Location of tilapia collection in Thailand

5.2.2.2 RNA extraction

For RNA extraction, a sample of spleen was removed from RNA later and blotted on paper to remove the excess amount of RNA later before transfer to a new tube filled with 1 ml of TRI reagent. The tissue samples were homogenized and added 100 µl of 1-bromo-3-chloropropane. Mixed thoroughly and left at room temperature for 15 min before centrifugation at 13,000 x *g* for 15 min at 4 °C. Transferred a top layer to a new tube, added an equal volume of isopropanol and precipitated at room temperature for 1 h. RNA pellets were collected by centrifugation at 13,000 x *g* for 15 min at 4 °C and discarded liquid component. Pellets were wahed with 1 ml of 75 % ethanol, left the tube at the room temperature for 5 min before centrifugation at 13,000 x *g* for 5 min, removed the ethanol and left dry. The pellet was dissolved with DNAse/RNase free water and measured the concentration with Nanodrop® ND-1000 (ThermoScientific, MA, USA). RNA was stored at -70 °C for cDNA synthesis.

5.2.2.3 RTqPCR

RNA (1,500 ng) samples were conversed to cDNA using Precision nanoScript2 Reverse transcription kit (Primer Design, Chandlers Ford, UK). Then the cDNA was diluted 7.5 times with RNAase/DNAse free water as a working solution and stored at -20 °C until use. A quality of cDNA was tested with β-actin primers before performing qPCR. A qPCR was conducted in 20 µl reaction with composed of 2 µl of cDNA, 1 µl of 10 mM forwards primer, 1 µl of 10 mM reverse primer, 10 µl of Luminaris Colour HiGreen qPCR master mix and 6 µl of DNAse/RNAse free water. qPCR was performed in triplicate with 8 cytokine gene targets; TNF-α, IL-1β, IL-6, IL-10, TGF-β, TLR3, IRF3 and Viperin. Details on qPCR protocol are described in chapter 3 (Table 3.3).

5.2.2.4 Statistical analysis

In terms of statistical analysis, t-test was used to compare copy numbers of mRNA between the two tilapia strains analysed. Moreover, gene expression levels in the various types of cultures and an interaction between species of tilapia and culture condition were conducted with ANOVA and multiple comparisons (Holm-Siduk). A significance level was considered at p-value<0.05 and generated a graph using GraphPad Prism version 6.0 (San Diego, CA, USA). A PCA (Principle Component Analysis) was conducted with R program version 3.5.2 (2018-12-20).

5.3 Results

5.3.1 Differential tissue distribution of selected cytokine and anti-viral mRNAs

This experiment aimed to study the distribution and relative intensity of selected innate immunity genes, related to pro and anti-inflammatory cytokine activities, in Nile tilapia and Mozambique tilapia. By using in-house Tilapia species (Institute of Aquaculture, University of Stirling), we analysed different organs relevant to immunity in fish in order to select a suitable tissue to use in further field experiments. Samples of head kidney, spleen and liver of Nile tilapia and Mozambique tilapia (n=9

were collected from the IoA tropical aquarium. cDNAs of samples were amplified with β-actin primers and observed on 1.5 % of agarose gel for quality testing. Gel electrophoresis showed a single clear band (144 bp) and similar density of the band across samples which was determined as adequate to perform qPCR (Fig. 5.5).

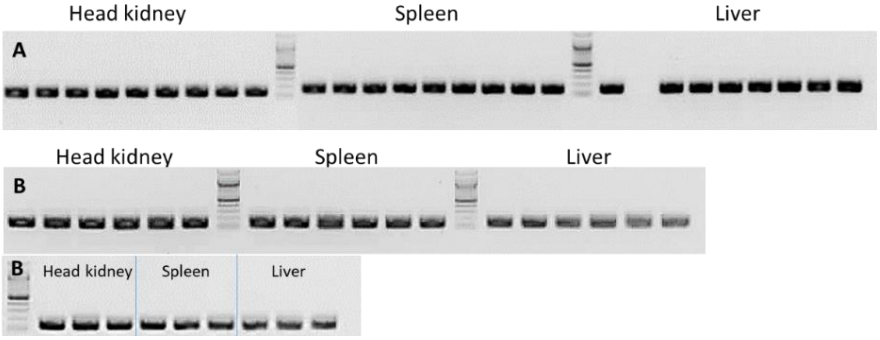


Figure 5.5 An agarose gel electrophoresis of the head kidney, spleen and liver with β-actin, a product size; 144 bp. A: Nile tilapia (n=9), B: Mozambique tilapia (n=9)

For Mozambique tilapia, qPCR analyses revealed that the amount of selected innate immunity mRNAs transcribed was highest in the spleen, followed by head kidney and liver (Fig 5.6A). An ANOVA and multiple comparison analysis showed significant differences in mRNA copy number among tissues for TNF-α, IL-1β, IL-6, IL-10 and TGF-β mRNAs. The amount of TNF-α, IL-1β, and IL-10 mRNA transcribed in spleen were significantly higher than that in head kidney and liver (P<0.05) whereas transcribed IL-6 and TGF-β mRNAs demonstrated significant differences between spleen and liver (P<0.05) but not between the spleen and head kidney.

In contrast, in Nile tilapia the transcription of the selected genes was mostly found in the head kidney (Fig. 5.6B). Pro-inflammatory cytokine expression (TNF-α, IL-1β, IL-6) was absent from the liver. The spleen showed the highest amount of TNF-α and TGF-β mRNA (no significant), however there was no detectable IL-1β or IL-6 mRNAs (Fig. 5.6B). An absence of these selected mRNAs in liver and spleen could be a result of qPCR error, technical mistakes or a very low level of mRNA transcribed in the tissues. To further explore the spleen mRNA repertoire TLR3, IRF3 and viperin mRNAs were analysed. Results show that TLR3, IRF3 and viperin were expressed in the spleen. Multiple comparison analysis of gene expression between Nile tilapia and Mozambique tilapia demonstrated that the level of TLR3 and viperin mRNAs of Mozambique tilapia were significantly higher than that of Nile tilapia. On the other hand, the level of IRF3 mRNA of Nile tilapia was higher than Mozambique tilapia (Fig. 5.7)

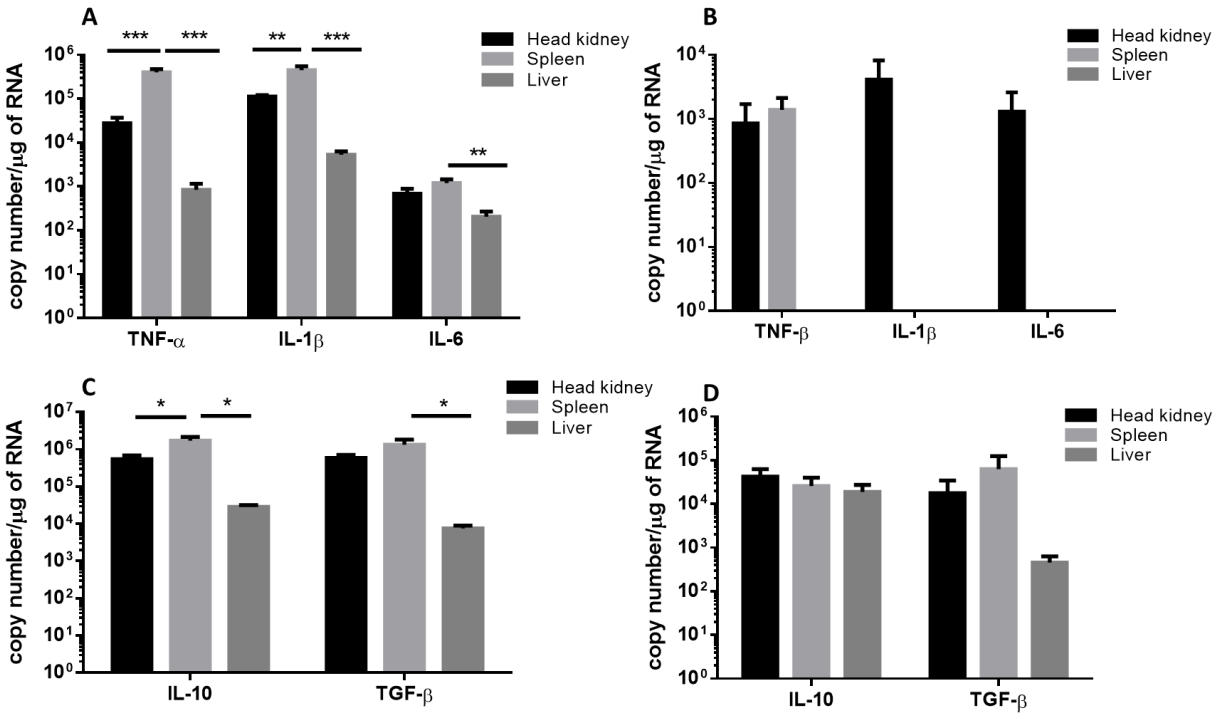


Figure 5.6 mRNA copy numbers for selected cytokines in head kidney, spleen and liver. A: pro-inflammatory cytokines mRNA of Mozambique tilapia and B: pro-inflammatory cytokines mRNA of Nile tilapia C: anti-inflammatory cytokines of Mozambique tilapia and D: anti-inflammatory cytokines of Nile tilapia. The data were analyzed using ANOVA and multiple comparisons with a level of significance; $P<0.05$ (n=6). Stars above bar denotes results significantly different.

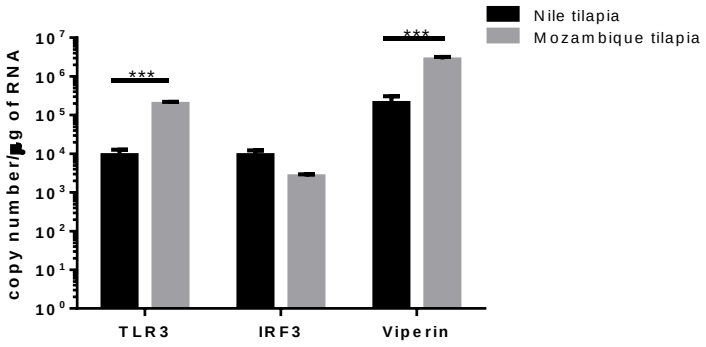


Figure 5.7 mRNA copy numbers for selected anti-viral targets in the spleen of Nile tilapia and Mozambique tilapia. The data were analyzed using t-test with a level of significance; $P<0.05$ (n=6). Stars above bar denotes results significantly different.

5.3.2 Interaction between genetic background and aquaculture system on tilapia innate immunity

5.3.2.1 Effect of genetic background on the expression of innate immunity-related genes

According to the previous experiment (5.3.1), the mRNA of target cytokines was able to detect in head kidney, spleen and liver, but the level of mRNA varied between the species of studied. Therefore, in this experiment, the spleen was a target organ as it is an important immune organ and has high mRNA level. Besides, spleen is more suitable for sampling during fieldwork than head kidney. Spleens of Nile tilapia (*O. niloticus*) and red hybrid tilapia (*O. niloticus x O. mossambicus*) were collected from various farming sites in Thailand and preserved in RNA later before RNA extraction. The cDNAs were synthesized and their quality were observed by PCR amplification with β-actin primers. There was a single clear band and even size presented on an agarose gel with a size of 144 bp. (Fig. 5.8).

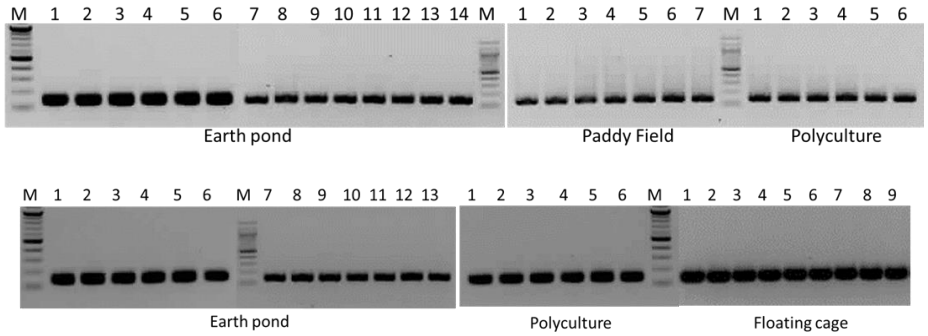


Figure 5.8 An agarose gel electrophoresis of spleen. The cDNA was amplified with β-actin primers, a product size; 144 bp. Top; Nile tilapia cultured in earth pond (monoculture) (n=14), paddy field (n=7) and earth pond (polyculture) (n=6). Bottom; red hybrid tilapia cultured in earth pond (monoculture) (n=13), earth pond (polyculture) (n=6) and floating cage (n=9)

In order to measure the level of gene expression, cDNAs of samples were amplified with selected primers of representative mRNAs relevant to innate immunity including; cytokines (TNF-α, IL-1β, IL-6, IL-10 and TGF-β), TLR3, IRF3 and viperin using an absolute RT-qPCR. Overall, red hybrid tilapia had a higher level of total gene expression than Nile tilapia. A significant difference between the levels of cytokine mRNAs between 2 species of Tilapia was observed. TNF-α, IL-1β and TGF-β mRNA abundances in red hybrid tilapia were higher than that of Nile tilapia ($p<0.05$). However lower levels of IL-6 and IL-

10 mRNAs were also measured (*P*<0.05). Moreover, a similar trend was found in the expression of TLR3, IRF3 and viperin mRNAs. The level of TLR3, IRF3 and viperin mRNAs in red hybrid tilapia were higher than Nile tilapia but only IRF3 mRNAs had a significantly higher *P*-value= 0.0405 (Fig 5.9). Statistical analyses are available in appendix 5.

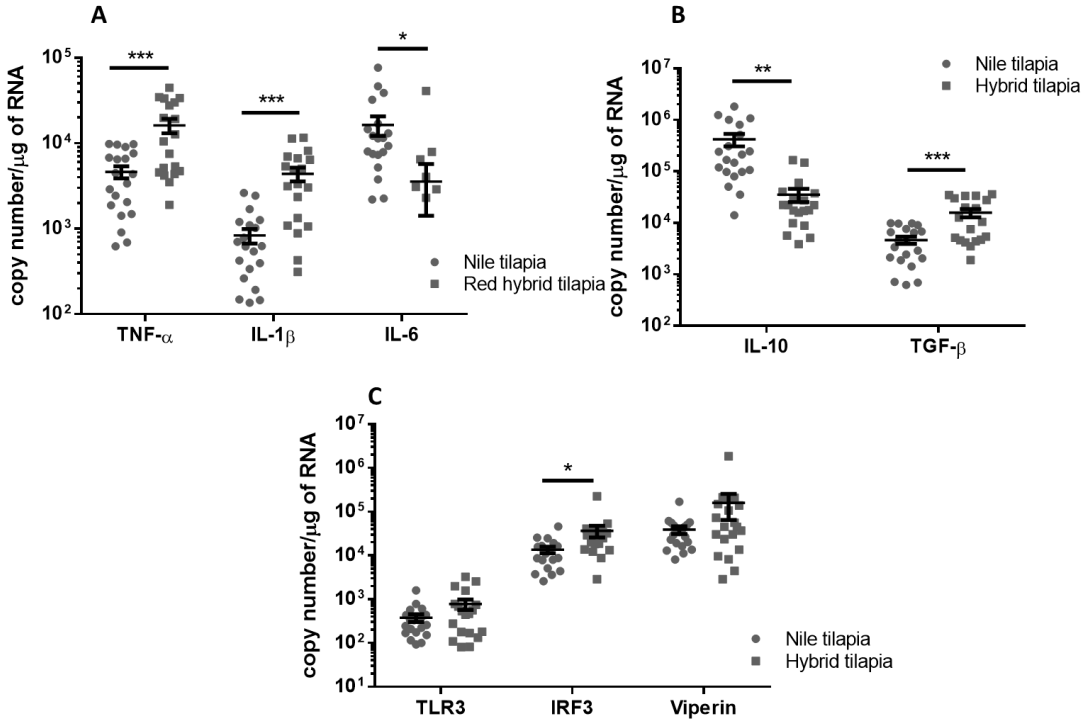


Figure 5.9 A comparison of mRNA transcription of innate immunity genes in the spleen of Nile tilapia (n=20) and red hybrid tilapia (n=19). The data were analyzed using unpaired t-test with a level of significance; P<0.05. Stars above bar denotes results a significantly different.

5.3.2.2 Effect of culture environment on the expression of selected innate immunity genes

Following the above analyses, spleens of Nile tilapia and red hybrid tilapia (*O.niloticus* x *O.mossambicus*) collected from various farming conditions in Thailand (see above) were further analysed. This analysis aimed to examine the impact of culture system on measured gene expression targets in the two tilapia species. A comparison of innate immunity genes expression between each culture condition was conducted with ANOVA and multiple comparisons (Holm-Sidak) (α=0.05) by using copy number data obtained from 5.3.2.1 and A PCA (Principle Component Analysis) was conducted to determine a distribution patterns. Tilapia samples were collected from 4 conditions;

earth pond (monoculture and polyculture), paddy field and floating cage. Only monoculture and polyculture system in earth pond where Nile tilapia and red hybrid tilapia samples were cultured in the same system due to logistical reasons.

A PCA graph revealed that Nile tilapia collected from various farming environments were grouped together on the left of the PCA, while the data of red hybrid tilapia were scattered. Red hybrid tilapia cultured in floating cage was separately clustered on the top right of the graph, wheares the samples from polyculture and monoculture were clutered on the bottom right. However, there was an overlapping of red hybrid tilapia cultured in monoculture and Nile tilapia cluster. Moreover, the PCA also presented a correlation between the selected genes (Fig. 5.10 and Appendix 5)

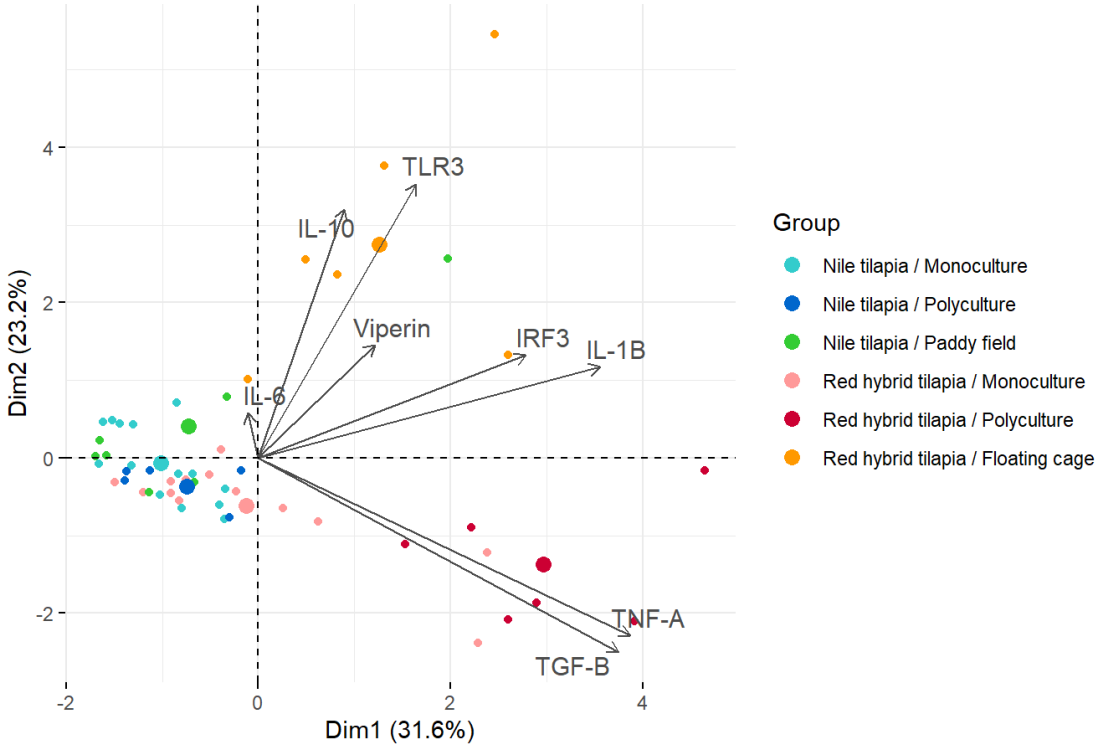


Figure 5.10 A PCA (Principle Component Analysis) of Tilapias cultured in various cultured condition in Thailand. Monoculture, Polyculture, Floating cage and Paddy field.

An ANOVA analysis of the mRNA transcriped of slected genes reveal that there was no significance different transcribed selected mRNAs between the culture conditions for Nile tilapia; Earth pond (monoculture and polyculture) and paddy field (Appendix 5). In stark contrast data for red hybrid tilapia highlighted an important effect of farming environment on cytokines (TNF-α, IL-1β, IL-10 and TGF-β) and TLR3 mRNA expression. A significant difference between monoculture and polyculture was observed for TNF-α, IL-1 β and TGF-β mRNAs. Monoculture and floating cage affected the measured abundance of IL-1 β, IL-10, and TLR3 mRNAs, and differences between polyculture and floating cage were presented for TNF-α, TGF-β and TLR3 mRNAs (Fig 5.11 and Appendix 5).

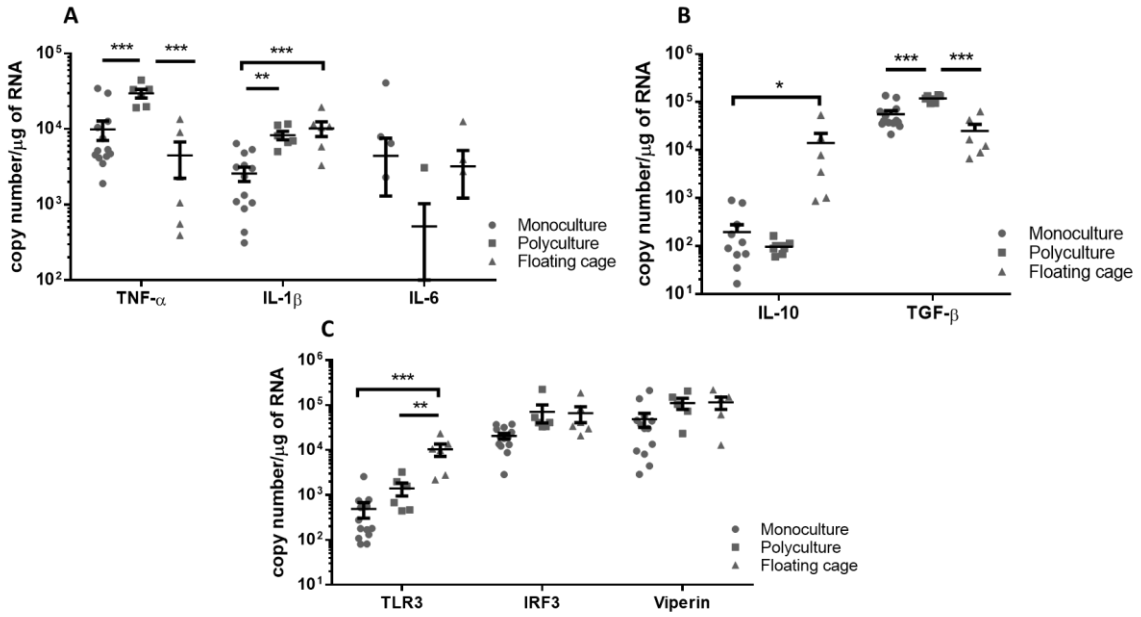


Figure 5.11 A comparison of mRNA transcription of innate immunity genes of red hybrid tilapia (*O.niloticus x O.mossambicus*) among culture condition. Monoculture (n=13), Polyculture (n=6) and Paddy field (n=6). ANOVA and multiple comparisons were tested with a level of significance; $P<0.05$. Stars above bar denotes results significantly different. A: pro-inflammatory cytokines B: anti-inflammatory cytokines

To explore differences between Nile tilapia and red hybrid tilapia cultured in the same environment; earth pond in monoculture system and polyculture system, we used two-way ANOVA (α=0.05) and multiple comparison analysis (Appendix 5) to examine the interaction between the strain of tilapia and environmental culture system. A comparison of innate immune mRNA markers between the 2 strains of Tilapia showed that the measured abundance of innate immunity-related mRNAs presented in the spleen of red hybrid tilapia was significantly higher than that of Nile tilapia, except IL-10 which was

lower (Appendix 5). An individual plots was used to determine an interaction between of Tilapia and culture coditions (monoculture and polyculture system in earth pond). This demonstrated that the culture system had a significant impact on the measured mRNA abundances of TNF-α, IL-1β, TGF-β, and IRF3 in red hybrid tilapia where measured mRNA abundances in polycultured fish were significantly higher than those in monoculture. In contrast, culture condition (monoculture and polyculture) has no significance effect in Nile tilapia (Fig.5.12).

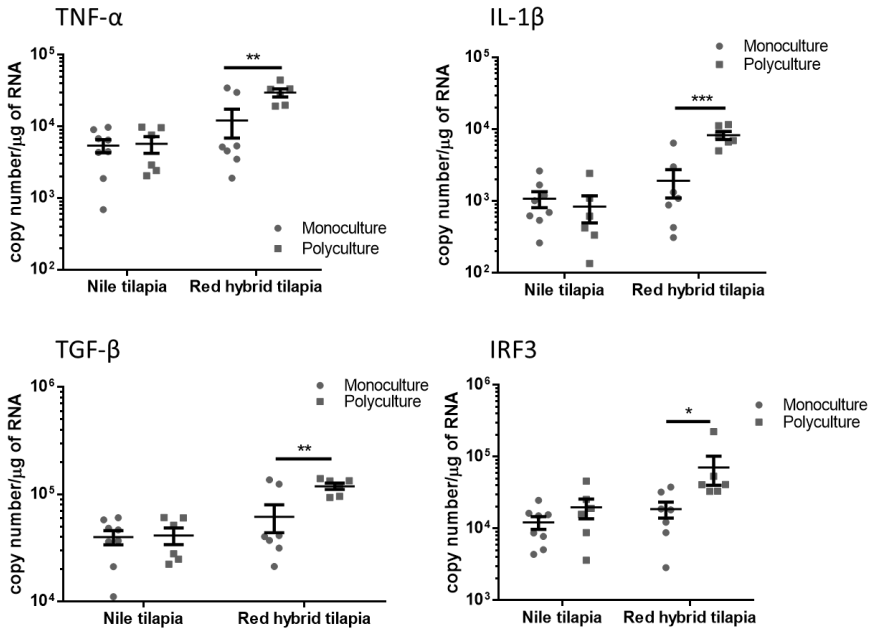


Figure 5.12 An interaction between the strain of tilapia (Nile tilapia and red hybrid tilapia) and culture condition (monoculture and polyculture). Both strains were cultured in earth pond. A copy number of mRNA transcription were analyzed using unpaired t-test (α=0.05). Stars above bar denotes results significantly different

5.4 Discussion

5.4.1 Immunity genes distribution on tissues

Head kidney (pronephros), spleen and liver are considered as central organs involved in the organisation of immunity in teleost fish. These organs have been extensively used as marker tissues to measure the expression of immune system relevant genes in many fish species under different experimental conditions (Roher et al 2011). In fish, head kidney is the central organ for hematopoiesis and plays a key regulatory function in immune system in terms of neuroimmunoendocrine interactions and antibody production (Whyte 2007). In parallel, spleen is involved in antigen trapping and degradation, and the antibody production process (review in Rauta et al 2012). Moreover, the size of spleen has been used as simple marker to investigate parasite infection (Lefebvre et al 2004). Liver has a role in metabolism and also produces complement and acute phase proteins. The size and colour of the liver has also been considered as a reliable biomarker for water quality for low dissolved oxygen in water and chemical pollution (Bruslé and Anadon 1996). Some examples of measuring an immune response of host against an infection on those tissues include; 1) mRNA expression of IL-1β and IL-10 of Atlantic cod (*Gadus morhua*) was highly up-regulated in spleen, followed by head kidney and liver after an intraperitoneal injection with poly I:C and formalin-killed *V. anguillarum*, however, a low level of IL-1β and IL-10 mRNAs was observed in control (Seppola et al 2008), 2) a similar result for IL-1β mRNA expression was reported in Japanese flounder (*Paralichthys olivaceus*) treated with formalin-killed *E.tarda* highlighting up-regulation of IL-1β mRNA in spleen and head kidney after 6 hours of treatment where the mRNAs in spleen were higher than head kidney in both treated and control conditions (Taechavasonyoo et al 2013), 3) the level of IL-6 mRNA of zebrafish (*Danio rerio*), at normal conditions was significantly higher in head kidney and spleen, followed by liver (Varela et al 2012), 4) IL-6 mRNA expression in grouper (*Epinephelus coioides*) was highly expressed in head kidney but very low level in spleen and liver under normal conditions (Chen et al 2012) which was similar to results obtained in this study for Nile tilapia and 5) the expression of TGF-β mRNA in goldfish (*Carassius auratus*) presented the highest level in spleen followed by liver and head kidney (Haddad et al 2008). These studies, as examples, highlight the ubiquitous use of head kidney, spleen and liver to measure the cytokine gene expression and their tissue distribution in a diverse range of fish species.

There is a problem with these studies as the immune system is dynamic and tissue distribution may change depending upon the physiological status of the individual fish. This adds a level of complexity to the interpretation of such studies. For example, it is more likely that immune responses are more localized rather than systemic therefore the measurement of cytokine in non-infected organs may not be representative of the actual response that is underway. In this study, mRNA of cytokines (TNF-α, IL-1β, IL-6, IL-10 and TGF-β) were detected in all target tissues (head kidney, spleen and liver) of Nile tilapia and Mozambique tilapia However, the level of mRNA transcript differed between the species. For further experiments, spleen was a target organ to examine an immune response of fish samples, it was not only because spleen is an important immune organ and has robust mRNA level but also more convenient for sampling in during field work than the head kidney.

5.4.2 Impact of the interaction between genetic background and environment; aquaculture system

In this study we examined the effect of different culture systems upon gene expression of selected components of the innate immune system of fish. We hypothesized that their immune system would be conditioned by the culture environment and this would also vary between different strains of fish. Therefore, spleen of Nile tilapia (*O. niloticus*) and red hybrid tilapia (*O. niloticus x O. mossambicus*) cultured in different farming environment in Thailand were collcted. The basal expression of healthy tilapia in each condition was examined through the mRNA abundance of selected genes related to pro- and anti-inflammatory response, and antiviral response (TNF-α, IL-1β, IL-6, IL-10, TGF-β, TLR3, IRF3 and viperin) transcript in the spleen. The basal gene expression of Nile tilapia and red hybrid tilapia was analysed to examine a variation of gene expression between the cultured strains and also analysed against their culture environment to determine an impact of cultue condition on baseline expression of the target genes.

In terms of the effect of the genetic background of tilapia on basal expression of the selected mRNAs we found that Nile tilapia and red hybrid tilapia which were collected from various environmental conditions presented significantly different amounts of cytokine mRNAs. mRNA abundance expressed as specific copy numbers for TNF-α, IL-1β, TGF-β, TLR3, IRF3 and viperin mRNAs in red hybrid tilapia were greater than those measured in Nile tilapia with the exception of IL-6 and IL-10 mRNAs that were significantly higher in Nile tilapia. Besides, it was found that the mRNA level of all studied genes in

Mozambique tilapia was higher than that expressed in the spleen of Nile tilapia. The expression of these genes has been reported in other species for example; a basal expression of IL-6 was high in the spleen of zebrafish (*Danio rerio*) (Varela et al 2012), but it showed a level of mRNA in grouper (*Epinephelus coioides*). Whereas, a high level of TGF-β mRNA transcription was found in the spleen of goldfish (*Carassius auratus*) (Haddad et al 2008). It is clear that between phylogenetically distinct groups, the pattern of mRNA expression might be different. On the other hand, the similarity expression pattern can be observed between species as they have the same immune system component which is conserved across teleost species. In addition, the basal expression of immunity genes depends upon the health status of the individuals that depend on the living condition, thus influence the immune response.

For this reason, the samples of Tilapia were analyzed against their living condition to examine the effect of environmental condition on basal expression of these selected genes. For Nile tilapia, reared in earth pond (monoculture and polyculture) and paddy fields, there were no significant differences between culture systems; earth ponds (monoculture and polyculture) and paddy fields. In contrast, the basal expression of the selected mRNAs in red hybrid tilapia was influenced by culture conditions. The level of mRNAs transcribed of TNF-α, IL-1β, IL-10, TGF-β and TLR3 showed a significant difference between culture conditions where floating cages has the highest level of mRNA, followed by polyculture and monoculture. Due to the high mRNA abundance measured for TLR3, which is a virus recognition receptor, it could be suggested that red hybrid tilapia cultured in floating cages might encounter with more viruses due to the continuous water stream. Culture environment of floating cage system in the river depends on water stream fluctuation and water quality which are difficult to control due to many farms hold a long the river which are different in farm scale and farming management. Therefore, farm practiced in downstream water tend to have lower water quality than farm practiced in upstream water. Whereas culturing in earth pond is possible to control such pressures. In this case, fish living in a high stress, through pathogen loading, the environment would likely demonstrate a modified innate immune response. Furthermore, the fish were sampled in April, when the highest temperatures are recorded in Thailand, fish tend to be more susceptible to disease than other periods such as *Streptococcus spp* which cause a disease outbreak during high temperature (Jantrakajorn et al 2014).

We further investigated the interaction between 2 strains of Tilapia and their culture environment; monoculture versus polyculture in earth ponds. Polyculture systems benefit in terms of improved water quality with better feed utilization and increase in total production (Wang and Lu 2015) and

adjust the ecological balance in ponds. Excess feed or aquaculture waste in monoculture systems is a valuable nutrient for phytoplankton which affect the oxygen dissolved in water, accumulation organic matter and also increase bacteria population mass in the pond. It leads to poor water quality resulting in a risk of stress and disease. It has been reported that culture environment affects the gut microbiome of Nile tilapia larvae which influences early host innate immunity, particularly in nutrient rich environments which promote microbial proliferation (Giatsis et al 2015). Tilapia can be cultured with many aquatic animals as major species or subordinate species such as shrimp (*Litopenaeus vannamei*), catfish (*Claris microcephalus*), or silver barb (*Barbonymus gonionotus*). In Thailand, tilapia is widely cultured with other aquatic species. For example, tilapia was cultured as subordinate species in white shrimp (*Litopenaeus vannamei*) pond in order to improve productivity, profitability, nutrient utilization and environmental friendliness of shrimp monoculture (Yuan et al 2010) or cultured with catfish(*Claris microcephalus*) to minimize waste in the production process (Lin and Yi 2003). However, behaviour and proportion of major and minor species load in culture practice must be addressed to achieve the maximum benefit.

In this study, monocultured Nile tilapia and red hybrid tilapia were collected from earth ponds in the same farm in order to maximize the similarity of culture environment and decrease variation. Whereas polycultured Nile tilapia and red tilapia were cultured in the same pond together with silver barb (*Barbonymus gonionotus*) and striped catfish (*Pangasianodon hypophthalmus*). Fish in monoculture systems were fed commercial feed whereas fish in polyculture systems fed upon both commercial feed and organic waste. Both conditions had a low seeding density. The investigation of interaction between strains of tilapia and their living environment demonstrated that, in the same environment, red hybrid tilapia has a higher level of basal expression than Nile tilapia, as almost all innate immunity mRNAs of red hybrid tilapia showed a larger copy number than in Nile tilapia, particularly when sourced from a polyculture system. As a result, monoculture and polyculture husbandry systems appear to have no impact on the innate immune response in Nile tilapia. However, polyculture has a significant effect upon basal levels of cytokines and receptors relevant to the innate immune response in red hybrid tilapia. The variation in genes expression profile of healthy fish is essential for the observation of changes during culture such as diseases, lack of nutritions or envirnonmental changes which correlation to health and behavior of fish.

5.5 Conclusions

Results of this study have shown that genetic background and culture environment have an impact upon the basal levels of mRNA for a number of innate immune-related genes. While further work should elucidate whether the basal levels of mRNA of the studied genes is linked to immune-state, results suggest that red hybrid tilapia is potentially more sensitive to culture conditions. On the other hand, Nile tilapia would appear to be less immune-sensitive under the different culture conditions studied in agreement with the success of this global aquaculture species

Chapter 6

General Discussion

Nile tilapia is an economically important fish with approximately 5.9 million tonnes (MT) of global production (FAO, 2019) and its production tends to increase every year. In order to support the increased market demand of tilapia, new robust strains of tilapia have been developed such as the GIFT strain generated using selective breeding by WorldFish. Breeding traits are mainly selected to meet farming and market requirements such as rapid growth, salinity tolerance, high meat quality, meat color and skin color. Surprisingly, although many studies have addressed tilapia and diverse aspects of its aquaculture including growth rate, nutrition, culture techniques etc., there is very limited data available on molecular and cellular immunity. In order to support health and production management of tilapia farming, it is necessary to understand the immune response in order to develop health management and welfare strategies. Although tilapia is considered relatively resistant to disease it remains to be detrimentally affected by significant disease issues such as those caused by *A. hydrophila* (Tipmongkolsilp et al 2012)*, S. innae* (Gaikowski et al 2014), *S. agalactiae* (Jantrakajorn et al 2014), *F. columnare* (Xu, D. et al 2015), *F. noatunensis* (Soto et al 2013) and Tilapia lake virus (*TiLV*) (Eyngor et al 2014; Jansen et al 2018).

Therefore, in this study we aimed to develop a platform to characterise the immune response of Nile tilapia at molecular level focusing on the initial activation of the innate immune response. The innate immune response is the first line of host defense against invading pathogens. Initial recognition and response are essential to survive in a microbe-rich environment that often characterizes certain types of tilapia aquaculture. In this thesis, a Nile tilapia macrophage primary cell culture was developed and characterized in chapter 2 in order to develop a model to determine PAMP-PRR interactions in chapter 3. In chapter 3, the expression patterns of key innate immunity genes was examined after macrophages activation with up-PGN or poly I:C which represent bacterial infection and viral infection. For the final experimental chapter (chapter 4), the expression pattern of these innate immunity characterized in macrophages was analysed tilapia cultured in Thailand. This allowed us to develop an insight into the effect of genetic background and culture environment on the immune system of tilapia.

In this experiment, samples of Nile tilapia (*Oreochromis niloticus*), red hybrid tilapia (*O. niloticus* x *O. mossambicus)* were collected from various types of farming system in Thailand.

6.1 Innate immune response and its cross regulation in Tilapia macrophages

The innate immune system is the critical defense system against invading pathogens of fish. The rapid non-specific target response activated upon PAMP-PRR recognition is essential to survival (Medzhitov 2007). Different environmental challenges, both abiotic and biotic, impact upon the immune response and likely shape the evolutionary development of the immune system across both the plant and animal kingdoms. From a pathogen recognition perspective, innate immunity relies upon PAMP-PRR interaction (Medzhitov 2007). PAMPs are conserved molecules representing essential functions in microbe biology and act as activators of the innate immune response of both plants and animals (Medzhitov and Janeway 1997). Important PAMP examples commonly used in research include the cell wall components LPS or PGN of bacteria, -glucan of fungi and nucleic acid of virus (MacKenzie et al 2010). These components are recognized by PRR on the immune cells of the host organism and in fish can categorized into 4 groups; TLRs, NLRs, CLRs and PGRPs (Boltana et al 2011). These receptors act as mediators to trigger specific signalling cascades in cells related to the immune response, such as macrophages, to activate the immune response.

In this study, to understand the interaction of PAMPs and PRRs of Nile tilapia, a macrophage primary cell culture derived from head kidney was induced with upPGN and poly (I:C) and we examined the expression of innate immunity-related genes in macrophages including; TLR2, TLR3, TLR8, TLR9, TNF-α, IL-1β, IL-6, IL-10, TGF-β, IRF3 and Viperin. The phylogenetic relationship of the target genes revealed conservation of innate immunity gene sequences in teleosts throughout evolutionary events, particular the TLRs. It has been reported that there are 18 TLRs in fish however the presence or absence of specific TLRs depends on the species (Zhang et al 2013). This has been suggested to be due to the impact of host-intrinsic factors or environmental changes (Solbakken et al 2017). A study of TLR evolution in teleost found that TLR9 is the most conserved gene and presents multiple copies in many species, followed by TLR3 and TLR8, whereas TLR2 is absent in several species of Gadiformes (Solbakken et al 2017). Moreover, Zhu et al (2013) reported conservation of TLR9 in Perciformes and

suggested that the evolution of immunity was driven by environment and the diversity of pathogens in a species-specific manner associated with the lifecycle of those fish.

An induction of TLRs (TLR2, TLR3, TLR8, TLR9) with poly (I:C) in Nile tilapia macrophages found that only TLR3 showed an up-regulation of TLR3 mRNA, while TLR2, TLR8 and TLR9 mRNAs showed a down-regulation. Poly (I:C) is an artificial dsRNA which is specifically recognized by TLR3, whereas TLR2, TLR8 and TLR9 are generally considered to recognize bacteria, ssRNA and unmethylated CpG DNA, respectively. Therefore, it may be unsurprising that only TLR3 showed a specific transcriptional response to this PAMP. Moreover, the up-regulation of TLR3 induced the transcription of both IRF3 and Viperin mRNAs which are critical downstream signalling components in the anti-viral response. These results demonstrate a specific recognition of TLR to PAMP, TLR3-dsRNA, and demonstrate the conservation of TLR3 function in triggering the antiviral signalling cascade. On the other hand, the antiviral immune response could also be due to the RLR pathway, which is another key pathway in RNA virus recognition that cooperates with TLRs to operate the immune response (Loo and Gale 2011). Thus, further studies are required to understand the contributions of TLR3 and RLR receptors such as RIG-1. However, this study using the macrophage model of Nile tilapia further illustrates the conservation of the antiviral response across the vertebrates.

In terms of bacterial infection, there are specific PAMPs that are recognized by the host PRR system such as those present on the bacterial cell wall; lipopolysaccharide (LPS) found in Gram-negative bacteria and peptidoglycans (PGNs) found in both Gram-negative and Gram-positive bacteria. It was reported that LPS which is a widely used PAMP to trigger immune responses in mammalian cell systems was not effective in fish (Iliev et al 2005). In some cases, fish become tolerant to LPS due to a chronic exposure to the pathogen in their living environment (Forlenza et al 2011). A similar report in mice demonstrated tolerance after repeated treatment with LPS (Erroi et al 1993). Further, it has been reported that the activation of rainbow trout cytokines was a result of PGN contamination in the LPS as upLPS (ultrapure) could not induce the production cytokines (MacKenzie et al 2010). Interestingly, fish have lost several of the key LPS recognition receptors (LBP, CD14, MD-2 and TICAM2) (Forlenza et al 2011), particularly TLR4 which is absent in all fish except zebrafish (Sepulcre et al 2009). However, a recent study in zebrafish reported that a possible intracellular receptor for LPS is caspy2 related to the inflammasome therefore, opening the door to new studies in the Teleost (Yang et al 2018) . A further study of Atlantic cod PRRs (Lin et al, manuscript under review) revealed that upPGN but not LPS induced the innate immune response of Atlantic cod through NLR receptor, NOD1. Importantly, cod

have lost almost all cell surface TLRs families suggesting that surface TLR signaling may not be essential to mount the innate immune response in fish.

The innate immune response of Nile tilapia to upPGN was examined through the level of inflammatory cytokine transcription. It was found that the upPGN induced the mRNA transcription of TNF-α, IL-1β, IL-6, and IL-10 but suppressed that of TGF-β. These cytokines are critical players in regulating the immune response and are classified into 2 groups; pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and anti-inflammatory cytokines (IL-10 and TGF-β). The pro-inflammatory cytokines promote inflammation and fever after infection by generation cell signalling cascade and triggering an adaptive immune response (Duque and Descoteaux 2014). While anti-inflammatory cytokines promote healing stage by suppressing the production of pro-inflammatory cytokines. However, there is a bi-directional control between pro-inflammatory cytokines and anti-inflammatory cytokines to stabilize the immunity balance as uncontrolled PRR or cytokines secretion can lead to severe tissue damage and chronic illness states (Srinivasan et al 2017).

TNF-α and IL-1β are pro-inflammatory cytokines, primarily secreted from ER of macrophages to the extracellular membrane as precursors. They are then released upon cellular activation and in the acute response, minutes response rather than hours, these precursors have an effective role in triggering the cytokine production cascade (Srinivasan et al 2017) and regulate their production in an autocrine fashion (Duque and Descoteaux 2014). In fish, specifically salmonids, this has been shown in trout macrophages where the rapid response of TNF-α to LPS or zymosan stimulation was a result of pre-formed membrane bound TNF-α peptides and the transcription of TNF-α was delayed behind the secretion of pre-formed membrane bound TNF-α peptides (Roher et al 2011). On the other hand, IL-1β is secreted as pro- IL-1β in the cytosol which is cleaved by CASP1 via a formation of inflammasome (a scaffold protein that induces CASP1 activity) (Kanneganti 2010). The active IL-1β has a role in stimulating lymphocytes and recruits macrophages to the site of infection generating both systemic and local responses to the infection (Dinarello 1996).

Both multifunctional cytokines have different regulatory pathways however they have cross-regulation and display overlapping functions to mediate inflammation. It was found that induction or suppression of TNF- α in cultures of synovial cells from rheumatoid arthritis (RA) patients has a positive feedback control on the production of IL-1 and other pro-inflammatory cytokines (Brennan et al 1989; Tseng et al 2018). Moreover, a recombinant TNF-α of rainbow trout was able to induce the expression of IL-1β, IL-8, TNF-α and COX-2 in rainbow trout macrophages (Zou et al 2003). A similar result study has been

reported in rainbow trout, a recombinant TNF-α3 induced the expression of IL-1β, IL-8, TNF-α, IL-6 and COX-2 (Hong et al 2019). However, IL-1β also has a role in induction the expression of pro-inflammatory peptides such as inducible nitric oxide synthase, COX-2, IL-6, and TNF-α (Dinarello 1996). Therefore, it can be suggested an insignificant up-regulation of TNF- α and IL-1β in macrophage of Nile tilapia after stimulation with PGN for 6 hours and 12 hours may due to a secretion of the precursor which was sufficient to regulate the immune response at an early state of activation where the transcription of the genes was delayed or a result of mycobacterium infection which was discussed in 5.2.

IL-6 acts in both stimulation and suppression of inflammation. IL-6 is triggered by TNF-α or IL-1β to stimulate inflammation. Whereas, IL-6 also has positive feedback in TNF-α activation as found in large yellow croaker, where the overexpression of IL-6 enhanced the expression of TNF-α (Zhu et al 2016). On the other hand, to promote anti-inflammatory activity, IL-6 can also inhibit the expression of TNF-α or IL-1β and stimulate IL-10. A study of IL-6 activity in orange-spotted grouper found that IL-6 has a vital role in naïve Th cell differentiation and promotes the production of antibodies (Chen et al 2012). Moreover, a recombinant IL-6 of rainbow trout was able to up-regulate the production of itself but down-regulate the production of TNF-α or IL-1β in macrophages (Costa et al 2011). This highlights the important role of IL-6 in the innate immune response of fish although relatively little is known about this cytokine in fish. In this study, the significant increase of IL-6 mRNA was found after PGN stimulation, approximately 43 times higher than control. As IL-6 has no precursor as TNF-α and IL-1β, it is regulated directly by de novo transcription. Thus, it is likely to be regulated by NLRs such as the NOD1 and NOD2 receptors, which directly reaction with PGN, and may highlight an important PAMP-PRR pathway in fish.

Anti-inflammatory cytokines (IL-10 and TGF-β), have a regulation role in reducing inflammation and promoting a healing stage by inhibiting a secretion of pro-inflammatory cytokines. TGF-β is an important cytokine, involved in cell proliferation, differentiation, migration and apoptosis under physiological and pathological conditions (Li et al 2006) and limit proliferation of T cell by inhibiting production of IL-2 (Li et al 2006; Yang and Zhou 2008). Whereas, IL-10 is mainly inhibit TNF-α, IL-1β, IL-6, GM-CSF and IFN-γ synthesis (reviewed in Fioranelli and Roccia 2014) and suppresses reactive O_2 radicals, phagocytosis and antigen presenting processes which indirectly limit Th cells function (Piazzon et al 2015). In this study, the level of IL-10 and TGF-β mRNA in Nile tilapia macrophages decreased at 6 hours after PGN stimulation and increased after 12 hours of activation which suggest a suppression

of pro-inflammatory cytokine at the early stage of activation and a cooperation between IL-10 and TGF-β. As it is known that production of IL-10 in monocytes is secreted shortly after a production of pro-inflammatory cytokines such as TNFa, IL-1, IL-6 and IL-8 (Zou et al 2003). Besides, Wei et al (2013) reported a relative activity between IL-10 and TGF-β in grass carp PBL, the level of TGF-β mRNA was up-regulation when activated with recombinant IL-10 but it was down-regulation when IL-10 was absent.

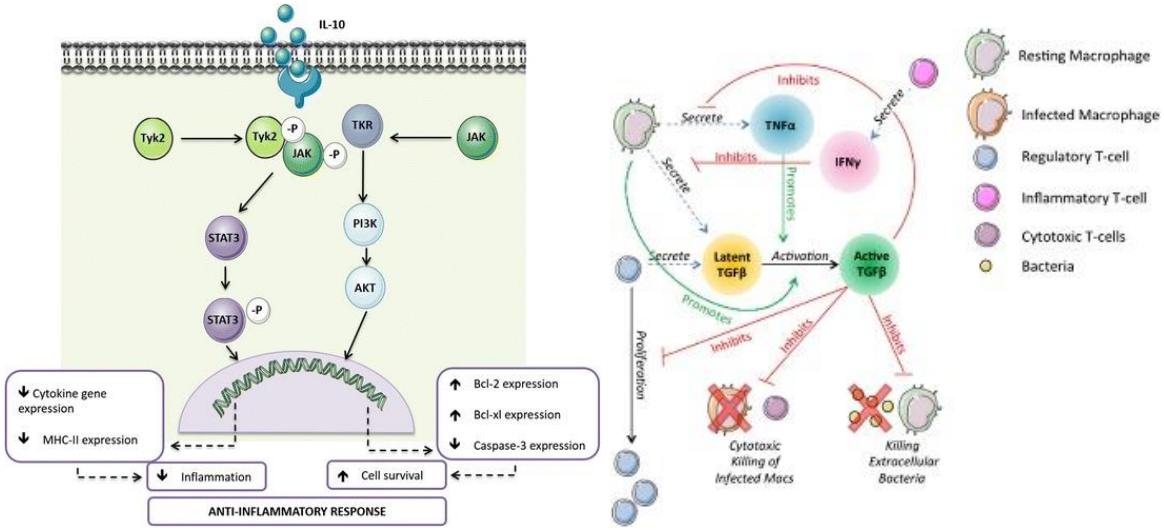


Figure 6.1 An overview of IL-10R signaling cascade (Lobo-Silva et al 2016) (Left) and TGF- β in anti-inflammation (Warsinske et al 2017) (Right)

It is believed that the expression pattern of pro-inflammatory and anti-inflammatory cytokines is regulated by a functional cross-talk among the cytokines. There are several studies that report the cross-regulation of cytokines in fish. For example; Wang et al (2016) reported an antagonistic role of TGF-β on the IL-1β expression in grass carp, a recombinant TGF-β could inhibit the production of bacterial-induced IL-1β mRNA in both in vitro and in vivo experiment, and TGF-β expressed delay after a rapid response of IL-1β to an infection. On the other hand, IL-1β also induced the transcription of itself mRNA and TGF-β mRNA in head kidney leukocytes (Yang et al 2014). However, TGF-β alone could not control the immune response, it was found that the expression of TGF-β in goldfish macrophages could induce by rTNF-α. Moreover rTGF-β together with rTNF-α were able to induce nitric oxide production in the macrophages but TGF-β alone has no effect on the macrophages (Haddad et al 2008). In terms of IL-10, the function of IL-10 has been characterized in goldfish (Grayfer et al 2011) demonstrated that recombinant goldfish IL-10 reduced the rapid response of monocytes to infection by suppressing the expression of TNF-α1, TNF-α2, IL-1β, CXCL-8 and SOCS-3, however IL-10 mRNA was

reduced by a recombinant TNF-α2. In contrast, IL-10 production can be induced by TNF- α in human peripheral blood mononuclear cells (Zou et al 2003). Besides, IL-10 could reduce IL-1β of Atlantic cod in both in vitro and in vivo study (Seppola et al 2008). These reports reveal an interplay between pro-inflammatory cytokines (IL-1β and TNF-α) and anti-inflammatory cytokines (TGF-β and IL-10) in the immune response during inflammation. The patterns observed in Nile tilapia in this study display a high degree to other fish species studied and by extension other vertebrates, however, there remains many aspects of fish immunology and cytokine regulation, particularly in respect to species specificities, environment and genetic background (wild versus domesticated/selected), that need further study.

6.2 Impact of *Mycobacterium* to the innate immune response of Nile tilapia

Mycobacterium is intracellular parasites that reside in host macrophages by interrupting the function of macrophages including phagocytosis and cytokines production to generate an appropriate inter-phagosomal environment for survival and growth (reviewed in Grayfer et al 2011). This may cause chronic disease without obvious clinical signs. A granuloma is a group of macrophages surrounded by lymphocytes and plasma cells or firmly bound epithelial cells (Puk et al 2017). The pathogens are arrested inside the granuloma in order to prevent nearby tissue from a further expansion of infection, the arrested bacteria do not multiple but can survive for a year inside the granuloma before presenting latent infection (Saunders and Cooper 2000). According to the characterization of macrophage primary cell culture of Nile tilapia (Chapter 2) and the examination of cytokines transcription in those macrophages (Chapter 3), there were noticeable observations which suggested a potential intracellular infection of the Nile tilapia used even though the fish had no clinical signs of infection. These observations included; 1.) Granuloma formation of macrophages and de-attachment of the cells during culture which is not observed in other species (rainbow trout, eel or salmon), 2.) Sustained levels of PGE_2 which were high in non-PAMP challenged cells and no increase of PGE_2 production after PGN stimulation despite the fact that PGE_2 concentrations in healthy cells/tissues are typically low and drastically increases after infection (Ricciotti and FitzGerald 2011). 3.) The balance between the expression pro-inflammatory and anti-inflammatory cytokines in the control cells which suggested that the cytokines were dysregulated as the mRNA transcription level had increased after PAMP stimulation but withouh showing a strong response. These results suggested a tolerance or chronic activation of the cell which could be due to *Mycobacterium* infection.

Due to a cross-regulation of immunity components, TNF-α and IL-1β have a role in the induction of COX-2 expression resulting in PGE_2 secretion. However, PGE_2 leads to an expansion of IL-10 which suppress TNF-α secretion in macrophages (Linke et al 2017). It was found that at the early stage of *M. tuberculosis* infection in mice, the aggregation of macrophages was promoted by TNF-α (Flynn et al 1995). PGE_2 was reported as a key mediator to induce production of regulatory T-cell (Treg) which are believed to have a crucial role in down-regulating the immune response to intracellular pathogens (*M. tuberculosis*) in humans. In this same study, the expansion of Treg cells resulted in a significant increase of IL-10 and TGF-β in human monocytes (Garg et al 2008). In terms of IL-10, it was found that IL-10 mediated macrophage deactivation causing a reduction of pro-inflammatory cytokines production and reactive oxygen species to promote a healing stage. However, during *M. tuberculosis*, IL-10 was found to damper the survival of bacteria inside macrophages by inhibiting IFN-γ, and cytotoxic T-cells (reviewed in Verma et al 2016). Moreover, Grayfer et al (2011) reported that *Mycobacterium* decreased the expression of IL-1β and TNFR1 in primary goldfish monocytes but increased the expression of IL-10 and TGF-β. These findings, are in agreement to the observed responses of Nile tilapia macrophages in this study. Furthermore, the detection of *Mycobacterium* DNA in the Nile tilapia macrophages increased when granuloma or cell mortality were observed suggesting that bacteria are arrested inside the granulomas and can escape when the macrophages die in the culture. In fish, infected macrophages restrict the growth of *Mycobacterium* but not necessarily eliminating them allowing the dissemination of the bacteria into a tissue. On the other hand, the bacteria utilize the macrophages as a carrier to disseminate into tissues (Clay et al 2007). However, the result of this study reveals a possible use for macrophages in culture to detect *Mycobacterium* in an early stage of infection by both facilitating isolation of bacteria and increasing growth rates of the mycobacterium which are difficult to grow in culture media.

6.3 Potential of innate immunity genes transcription levels for use as health status biomarkers

In this thesis, we aimed to characterise interaction between PAMPs and PRR in Nile tilapia in order to provide a knowledge platform for understanding the molecular regulation of the immune response. We developed a model of macrophage primary cell culture and using the model determined patterns of innate immune responses in tilapia macrophages. In addition, the absolute RTqPCR assays developed were used to explore differences in tissue expression of these inflammatory genes and their relationship to genetic background and culture environment. Biomarkers are characteristics that useful indicators of normal biological processes, pathological processes or pharmacological responses to a therapeutic intervention. Biomarker approaches to assess health condition and disease development in humans range from reductionism studies of disease-specific proteins to holistic approaches including biological networks to discriminate diseases (Dudley and Butte 2009). In teleosts, several molecular biomarkers have been identified and applied. For example; macrophages of Atlantic salmon were used as monitoring biomarkers upon bacterial and viral infection; *Piscirickettsia salmonis* (Rise et al 2004) and ISAV (Workenhe et al 2009). Moreover, gill tissues have been used to mornitor thermal stress in Pacific salmon (Akbarzadeh et al 2018), it was found that the expression of genes involved in chaperoning and protein rescue, oxidative stress and biossysthesis in gill tissue of Pacific salmon respond to an increasing temperature. These studies suggest a potential development of biomarker and its effective in fish.

In this study, using a differential gene expression approach we were able to map different values for each of the target genes and associations to culture environment in both macrophages primary cell model and tissue samples. Due to the homogenization of cell culture condition, the macrophage cells in the same well tend to have similar behavior under the experimental conditions, but still reflect the physiological condition of their original fish. This results in a decrease in inter-sample variation as would be expected using this reductionist approach. In terms of tissues, the basal expression of the genes and their distribution was dependent upon the physiological status of the individual fish even growing in the same environment. This adds a complexity to resolve the data unless the number of fish is increased. On the other hand, discrimination of coping style or behaviour of fish can be used to decrease a variation of genes expression study, as the expression of genes in response to stress is influenced by a different coping styles of fish (MacKenzie et al 2009). It suggests localization of the

immune response rather than systemic circulation, thus the measurement of cytokine in non-infected tissue may not reflect an actual activity of the fish. However, even though the expression of the genes differs among tissues and species, the pattern of the immune response is similar due to a similarity of immune system components and functional conservation of the immunity genes.

In terms of the studied genes, it was found that the health condition of the macrophages could be revealed through the expression of innate immunity genes together with PGE_2 production and observation of the cell behaviour, even though this may be limited to a *Mycobacterium* type infection. In farms, it is difficult to assess the health status of fish as there are various factors that cannot controled such as pathogens and environmental conditions and fluctuations. Furthemore, some fish strains might be more amenable to environmental and disease challenges. With the interaction of these factors, to a ascertain the condition of the fish, particularly when the fish is under subclinical infection might be very challenging. This leads to difficulties in data variation and interpretation. However, the examination of basal expression of the innate immunity related genes in Nile tilapia and red tilapia cultured in various conditions demonstrated an effect of culture condition on the expression of red hybrid tilapia immunity genes while the culture condition has no impact on the basal expression of genes in Nile tilapia. Although, this cannot illustrate a positive or negative health condition it reveals an impact of different culture conditions on red hybrid tilapia which should be further examined.

In order to increase a potential application of these innate immunity genes as health biomarkers, it is better to have more data of basal expression and also the data of genes expression in moribund fish of each culture system for comparsison. Besides, other points that would need to be addressed are the size of the fish as the immunity of fish is related to its size, and sampling period as different period tends to have different effect to the fish such as temperature and diseases. This will expand the efficiency of the marker to reflect the actual health status of fish which will benefit Tilapia health management and production.

Chapter 7

General Conclusions and Future perspective

7.1 General conclusions

A global conclusion of this study can be made that the macrophage primary cell culture derived from the head kidney of Nile tilapia is an effective model to determine the interaction of host PRRs upon exposure to PAMPs. This model will facilitate further studies of the immune response in the Nile tilapia. In order to fully exploit this cellular model, a number of further considerations should be taken into account including: 1.) Culture conditions are critical factors that impact upon the basal expression of innate immune response mRNAs and these conditions vary between fish, 2.) The source of the macrophages which provides a proper number of cells for study. 3.) Fresh tissue, that should be collected from same source throughout the experiment and the fish have no infection.

In this study, the basal expression of studied mRNAs revealed a chronic infection which was caused by an intracellular bacterial infection in the stock fish held in our aquarium system (*Mycobacterium*). Although concerning in view of results obtained, lack of inflammatory activation, our results also illustrate the potential of the macrophage primary cell culture to detect *Mycobacterium* at an early stage of infection. The model both facilitates the isolation of bacteria and increases growth rates of the *Mycobacterium* which are difficult to grow in culture media, highlighting an innovative and practical use for this model system.

In terms of the innate immune response in the Nile tilapia, the phylogenetic and functional analysis highlight that the selected innate immunity genes are highly conserved across different taxa and extant teleosts, and the PAMP-PRR recognition are also strongly conserved. The immune response is often localized rather than systemic therefore the measurement of cytokine mRNAs in non-infected tissues may not reflect the actual immunological status of the fish. Taking this into account our results show that the expression of the target mRNAs differs among tissues and species. Nevertheless, the pattern of the immune response is similar highlighting the functional conservation of the immune system in the fishes.

Finally, the immune system of fish were conditioned by their culture environment and genetic background. The basal transcription profile of innate immunity related genes of Tilapia depends on culture environment and their strain, however an interaction between these factors were relevant to the basal expression of target genes. As monoculture and polyculture husbandry systems have no impact on the innate immune response in Nile tilapia but polyculture has a significant effect upon basal levels of cytokines and receptors relevant to the innate immune response in red hybrid tilapia. The variation of the basal expression of healthy fish will facilitae an observation of fish health status due a change in culture condition such as to diseases, nutritions or environmental problem which reflect to health and behavior of fish.

7.2 Future perspectives

The current study suggests an application of macrophage primary cell culture model to determine the innate immune response of host upon the infection of the pathogen, which will reduce the experimental scale and number of animals sacrificed. This follows the NC3Rs principles. However, proper culture conditions and the quality of fish samples must be considered to reduce the observed variation in results obtained as a fresh tissue is required in every experiment. To clarify a PAMP-PRR interaction of Nile tilapia, naïve non-infected Nile tilapia are needed to investigate the function of the macrophages; phagocytic activity or PGE_2 production and the level of gene expression during the inactive stage compare with an activated stage. Moreover, further studies on RLR and NLR pathway are required to understand the specific contributions of PRRs upon pathogen exposure and infection. Besides, to increase a capability of the model as a biomarker, the larger samples information such as a basal expression of fish in different culture environment at different period of times and also the samples of moribund fish during disease outbreak, are recommended to reflect the actual health status of fish which will benefit tilapia health management and production.

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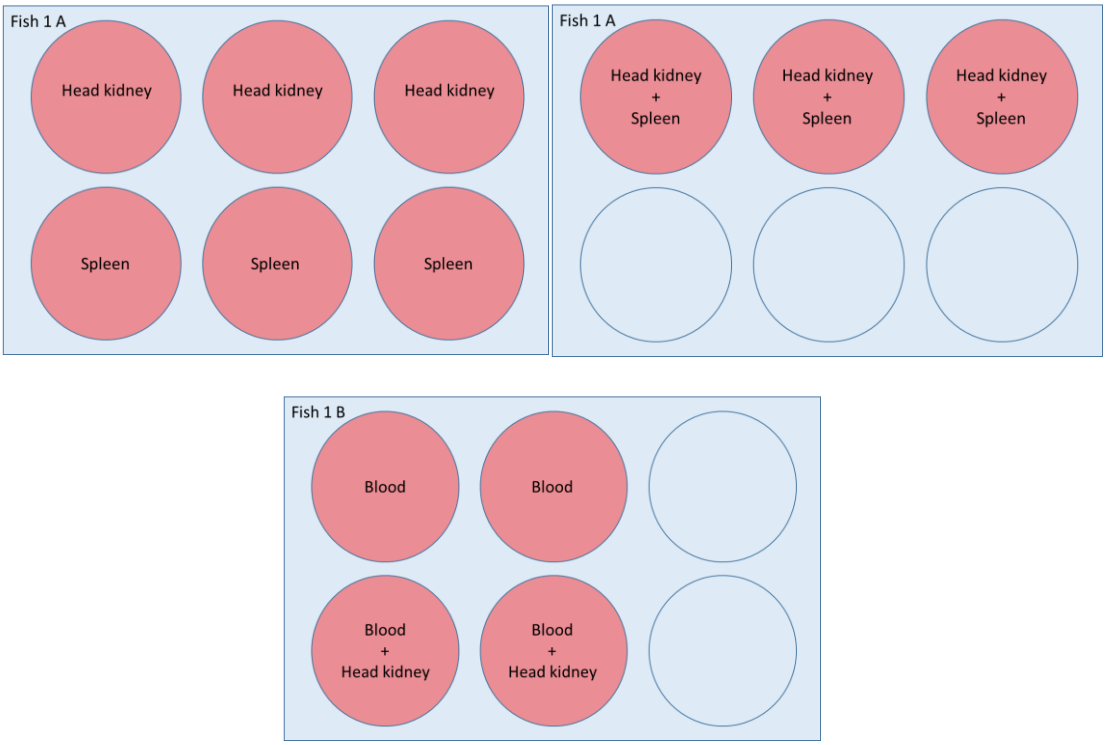
Appendices

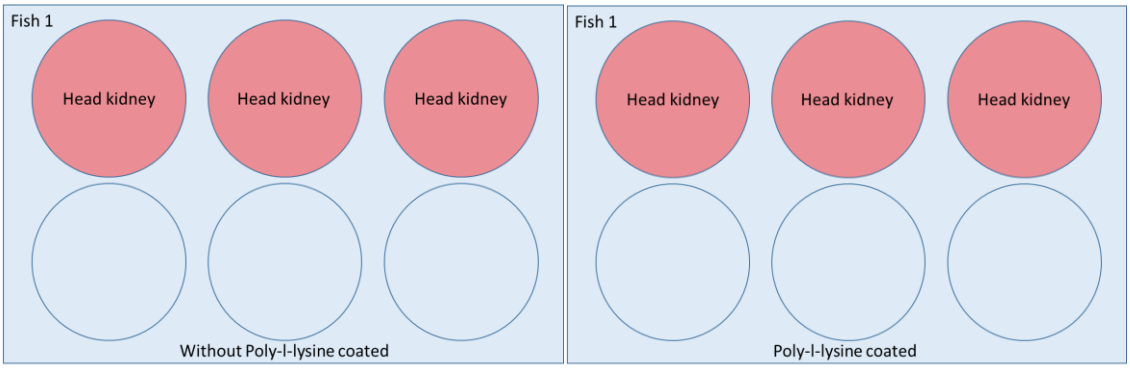
Appendix 1 Experimental plate designs

Appendix 1 showed the experimental plate design during optimization of culture condition for macrophages primary cell culture of Nile tilapia. Each experiment was performed in 3 biological and 3 technical replications; 3 fish samples/experiment and 3 replications of cells were seeded/condition. However, the figure presented, are experimental plate design of 1 fish.

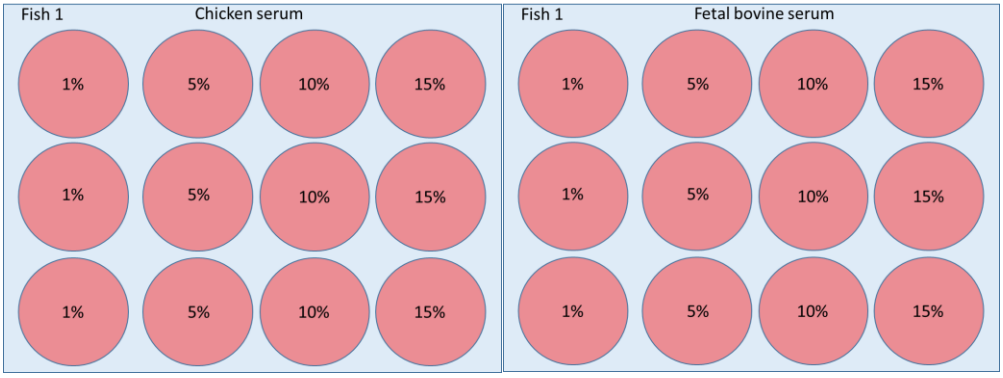
1. Macrophages cell sources



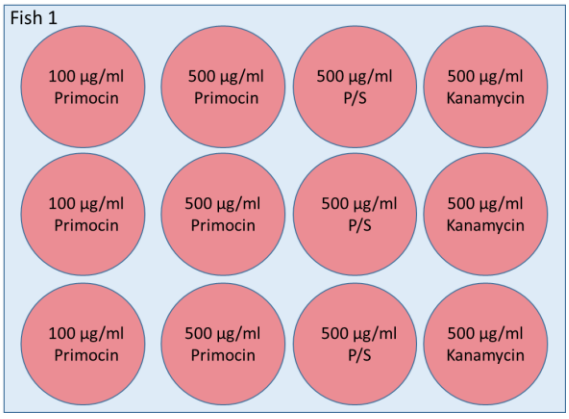
2. Effect of Poly-l-lysine (PLL) on macrophages-like cell differentiation



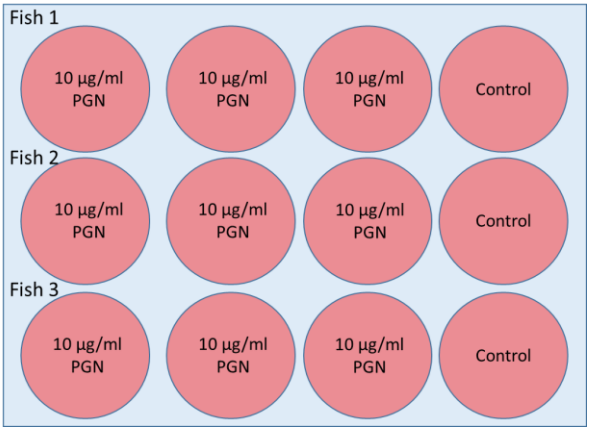
3. Effect of different types and concentration of serum on macrophage primary cell culture



4. Effect of antibiotics and their concentration on macrophage primary cell culture



5. Effect of ultra-pure Peptidoglycan on macrophages



Appendix 2 Primers and sequencing

Genes	Primers Sequences	Product Size	T_a	Product sequences
TNF-α	>F CAGGATCTGGCGCTACTCAG >R TAGCTGGTTGGTTTCCGTCC	184 bp	60 ˚C	5’ CAGGATCTGGCGCTACTCAGAGTCTATGGGAAGC AGCTCCACTCTGATGAGCGCCCTGAGGTCGGCGTGC CAAGACACCATTCAGGATAGCTTCTCAGACCACGGCT GGTACAACGCCATTTACCTGGGCGCTGTGTTTCAGCT GAACGAAGGAGACACGCTGTGGACGGAAACCAACC AGCTA 3’
IL-1β	>F TGAGAGCCTACTTTAGGATTCTGC >R GCGGCTATTACAACCAATGCT	150 bp	59 ˚C	5’ TGAGAGCCTACTTTAGGATTCTGCAAATAAATAAT ACCCTAATCTGCTGTACTTCCTACCTACAGATCTCCTA CCAAACTTGGGATATAATTTGGGAAATTACTCTTCAT CAGTAATCCTTTCACTTGAGCATTGGTTGTAATAGCC GC 3’
IL-6	>F CTGAGTGAGGGGAAAAGAGC >R AGGAGTGTCAAAACCATCCAG	148 bp	61 ˚C	5’ CTGAGTGAGGGGAAAAGAGCTGTTTGTAGAATGG AAAAGCGAGTGAACCAATCAGCAGATACCAGTTGTTA AAAAGACAAATAATGCTCAAATTATATTCAATTATGAA ATAATAGCTCAGGGAAAACTGGATGGTTTTGACACTCC T 3’
IL-10	>F CTCAGATGGAGAGCAGAGGTC >R CTTGATTTGGGTCAGCAGGT	134 bp	60 ˚C	5’ CTCAGATGGAGAGCAGAGGTCTATACAAGGCCA TGGGAGAGCTGGACATATTGTTTAACTACTTTGAGA CGTACCTGGCTTCTAAACGACACAGACAATAACCTC TGCTCGAAGACCTGCTGACCCAAATCAAG 3’

TGF-β	>F GAGATCCCTGCCAACTTGCT >R TCCCCGACGTTACTCCGTAT	230 bp	60 ˚C	5’ GAGATCCCTGCCAACTTGCTATCACTCTACAACAG CACCAGTGAGATTCTGCTGGAGAAGCAGGATGAGG AGCAGAAAATCATCCCCAGAGAACAAGAGGAGGAG GAATACTTTGCCAAGGTGCTTAACAGGTTCAACATG ACCACAAAAAATGACACAAACATCAACTACAAGCCC AAAGTCATCTCAATGAGCTTCAACATCTCTGAGATAC GGAGTAACGTCGGGGA 3’
Viperin	>F ATCAACTTCTCTGGCGGA >R AGATAGACACCATATTTCTGGAAC	161 bp	56 ˚C	5’ AGATAGACACCATATTTCTGgAACCATTCTTCTTTGA TCATGCTTCCATTGCTGACAATGCTGACACTTGGGAGC TGGAGGTCCTGTTTGCAGTACTGGACCAATTTCCCCAC AAACTCTCCCTTACCATGCAGGAAGGGCTCTCCGCCAG AGAAGTTGAT 3’
TLR3	>F CTGTCCGTCACTCCGAAACA >R CCGGGATTGATCTGCGCTAT	108 bp	59 ˚C	5’ CTGTCCGTCACTCCGAAACATCACCAACAAGACCTT AGCGTCTCTTGCAGATTCACCAGTCAGATGGCTAAATC TCATAAATACAAATATAGCGCAGATCAATCCCGG 3’
TLR2	>F TCTGGGCTATCCTTCCCCAA >R TCGCAGATGTAGCTGTCCAC	221 bp	60 ˚C	5’ TCTGGGCTATCCTTCCCCAATCTACAAACCCTGACC ATCCAATCAAATACCCTGAACATGTTTGGCAAAACAGA CCTCCAGTCATACCAGCGACTCGAGAACCTCCAGGCTG GTCAGAATAAGTTTGTCTGCTCCTGTGACTTTGTCAGCT TTGTCCAGTTGGAACTCCGAGGAGGTGGCGGTATAGAA CTGACAGATGGAGTGGACAGCTACATCTGCGA 3’
TLR8	>F TCTGAGTGGGTGATGAGCA >R TGTACTGGATGCTCTGGGTG	137 bp	61 ˚C	5’ CTCTGAGTGGGTGATGAGCAATCTGCGGGTGCAACT GGAGGAAAAGGGAGACAAGTATCATCCACTGTGTCTGG AAGAGAGGGACTGGCCCCTAGGAGTCCCACTGGTGGAC AACCTCACCCAGAGCATCCAGTACA 3’
TLR9	> ACCTTCCTGGACCTCAGTCA > TGGCATGCAGGGTGAGATTT	178 bp	60 ˚C	5’ACCTTCCTGGACCTCAGTCACAATCGGATCAGTTATAT TCCTGAAGATTTCTTTAATAATGCAAAGTCCTTAAAATAT TTGTATCTCAGTCACAATCAGATCAAAGAGTTGAACCGA GAGCATCTTCCTGTCCTCTTTAAAAATGACACTCGCCTTG AAAATCTCGCCCTGCATGCCA 3’
IRF3	>F GGTACGACACATCAGCGTGC >R CTGGCAACATAGAGCAGCAGTA	183 bp	60 ˚C	5’ CTGGCAACATAGAGCAGCAGTACAGTCACATGTG AGATCACAATGGTGAAAAGGCCATTTTGATCATGTA GTAGATTCAGCAGAAGAATACTACCCATTAATGTTTC AATAAAGTTTTCAATTCGATTAATCTGCGGTATCGAA CAGTGTGATTATCTGCACAGCACGCTGATGTGTCGTACC 3’

B-actin	>F GCTACTCCTTCACCACCACAG >R CGTCAGGCAGCTCGTAACTC	144 bp	61˚C	5'GCTACTCCTTCACCACCACAGCCGAGAGGGAAATC GTGCGTGACATCAAAGAGAAGCTGTGC TACGTCGCCCTGGACTTCGAGCAGGAGATGGGCACC GCTGCCTCCTCCTCCTCCCTGGAGAAGA GTTACGAGCTGCCTGACG 3'

Appendix 3 qPCR standard

Co-efficient of determination (r^2), efficiency and sensitivity for the target genes generated from the standard curve. Efficiency was calculated using the formula, E =10(-1/- slope) . E value of 2.0 is equivalent to 100% efficiency. N represent the number of qPCR run for each gene to make standard curve while sensitivity is the lowest copy number detected by qPCR.

Genes	R^2	Efficiency	N	Sensitivity
TNF-a	0.9816	2.286899	3	10^2
IL-1b	0.989	2.333816	3	10^2
IL-6	0.9886	1.889048	3	10^2
IL-10	0.98933	2.142214	3	10^2
TGF-a	0.98116	2.286899	3	10^2
IRF3	0.9478	1.898913	3	10^2
Viperin	0.965	1.892681	3	10^2
TLR2	0.8256	2.095538	3	10^2
TLR3	0.9782	2.332289	3	10^2
TLR8	0.9657	1.76324	3	10^2
TLR9	0.9962	1.879751	3	10^2

Appendix 4 Distance score

TNF-α

Nile_tilpapia									
Human	143.00								
House_mouse	146.00	47.00							
Zebra_fish	133.00	156.00	161.00						
Atlantic_salmon	132.00	147.00	152.00	145.00					
Rainbow_trout	96.00	150.00	140.00	146.00	132.00				
Guinea_pig	143.00	40.00	48.00	155.00	147.00	151.00			
Nile_tilapia_B-actin	186.00	184.00	185.00	205.00	198.00	199.00	184.00		
Common_carp	3.00	143.00	146.00	131.00	130.00	96.00	143.00	187.00	
Xenopus	157.00	141.00	142.00	159.00	167.00	170.00	140.00	193.00	155.00

IL-1β

Zebra_fish									
Rainbow_trout	373.00								
Nile_tilapia_B-actin	468.00	450.00							
Nile_tilapia	393.00	391.00	490.00						
Medaka	418.00	398.00	466.00	345.00					
Human	415.00	393.00	500.00	407.00	430.00				
House_ouse	415.00	395.00	500.00	403.00	420.00	147.00			
Guinea_pig	417.00	408.00	504.00	401.00	432.00	146.00	149.00		
Common_carp	249.00	356.00	497.00	394.00	416.00	406.00	400.00	407.00	
Atlantic_salmon	372.00	22.00	449.00	396.00	396.00	384.00	389.00	397.00	358.00

IL-6

Nile_tilapia									
Human	218.00								
Mouse	229.00	151.00							
Zebra_fish	221.00	241.00	251.00						
Nile_tilapia_B-actin	269.00	266.00	274.00	259.00					
Rainbow_trout	192.00	238.00	239.00	204.00	236.00				
Guinea_pig	222.00	154.00	162.00	254.00	261.00	229.00			
Atlantic_salmon	191.00	236.00	237.00	205.00	239.00	13.00	228.00		
Puffer_fish	162.00	223.00	242.00	197.00	224.00	174.00	234.00	177.00	
Chimpanzee	217.00	1.00	150.00	240.00	265.00	237.00	153.00	235.00	222.00

IL-10

Nile_tilapia									
Guinea_pig	172.00								
Human	169.00	37.00							
Mouse	170.00	55.00	54.00						
Zebra_fish	145.00	175.00	163.00	175.00					
Puffer_fish	106.00	179.00	167.00	178.00	140.00				
Atlantic_salmon	118.00	171.00	166.00	174.00	124.00	120.00			
Common_carp	143.00	173.00	166.00	176.00	52.00	144.00	126.00		
Rainbow_trout	116.00	170.00	169.00	174.00	123.00	120.00	16.00	121.00	
Nile_tilapia_B-actin	199.00	205.00	205.00	207.00	202.00	196.00	189.00	201.00	189.00

TGF-β

Nile_tilapia									
Human	226.00								
Zebra_fish	199.00	231.00							
Atlantic_salmon	194.00	226.00	175.00						
Common_carp	203.00	243.00	196.00	166.00					
Guinea_pig	219.00	58.00	225.00	213.00	247.00				
Nile_tilapia_B-actin	350.00	333.00	348.00	339.00	366.00	344.00			
Mouse	289.00	294.00	286.00	274.00	307.00	287.00	337.00		
Chimpanzee	226.00	2.00	232.00	225.00	243.00	59.00	333.00	294.00	
Rainbow_trout	190.00	232.00	173.00	38.00	161.00	221.00	350.00	283.00	231.00

IRF3

Human									
Mouse	267.00								
Zebra_fish	542.00	547.00							
Atlantic_salmon	533.00	532.00	374.00						
Common_carp	535.00	540.00	177.00	394.00					
Rainbow_trout	540.00	533.00	377.00	34.00	394.00				
Guinea_pig	283.00	280.00	548.00	534.00	545.00	539.00			
NIle_tilapai_B-actin	549.00	571.00	535.00	533.00	510.00	522.00	573.00		
medaka	529.00	530.00	370.00	299.00	387.00	307.00	533.00	500.00	
Nile_tilapia	526.00	526.00	362.00	271.00	375.00	278.00	518.00	526.00	217.00

Viperin

Human										
Mouse	240.00									
Zebra_fish	504.00	502.00								
Atlantic_salmon	498.00	502.00	363.00							

Common_carp	498.00	494.00	168.00	384.00						
Rainbow_trout	502.00	501.00	366.00	32.00	385.00					
Guinea_pig	249.00	257.00	501.00	512.00	503.00	513.00				
Nile_tilapia_B-actin	543.00	531.00	508.00	525.00	492.00	522.00	541.00			
Medaka	498.00	499.00	362.00	289.00	376.00	297.00	491.00	519.00		
Chimpanzee	62.00	233.00	503.00	492.00	495.00	493.00	246.00	537.00	488.00	
Nile_tilapia	486.00	488.00	350.00	261.00	360.00	268.00	481.00	503.00	203.00	480.00

TLR2

Nile_tilapia									
Human	474.00								
Mouse	475.00	223.00							
Zebra_fish	503.00	468.00	482.00						
Common_carp	494.00	465.00	490.00	196.00					
Rainbow_trout	368.00	486.00	469.00	469.00	466.00				
Guinea_pig	484.00	247.00	284.00	472.00	471.00	463.00			
Nile_tialpia_B-actin	728.00	699.00	692.00	700.00	683.00	666.00	680.00		
Atlantic_salmon	374.00	484.00	471.00	461.00	459.00	59.00	463.00	667.00	
Xenopus	599.00	568.00	562.00	584.00	581.00	584.00	557.00	717.00	590.00

TLR3

Nile_tilapia									
Mouse	526.00								
Zebra_fish	445.00	507.00							
Common_carp	433.00	503.00	164.00						
Rainbow_trout	360.00	485.00	415.00	406.00					
Guinea_pig	547.00	228.00	524.00	513.00	518.00				
Nile_tilapia_B-actin	692.00	681.00	667.00	675.00	686.00	681.00			
Human	524.00	193.00	517.00	504.00	504.00	148.00	668.00		
Atlantic_salmon	352.00	490.00	415.00	406.00	32.00	524.00	688.00	511.00	
Xenopus_laevis	535.00	462.00	503.00	510.00	527.00	445.00	717.00	444.00	532.00

TLR8

Nile_tilapia									
Mouse	122.00								
Atlantic_salmon	87.00	117.00							
Common_carp	105.00	133.00	97.00						
Nile_tilapia_B-actin	182.00	209.00	189.00	193.00					
Human	121.00	31.00	114.00	131.00	205.00				
Xenopus	126.00	106.00	127.00	145.00	189.00	110.00			
Chimpanzee	121.00	31.00	114.00	131.00	205.00	0.00	110.00		
Zebra_fish	102.00	143.00	87.00	46.00	187.00	139.00	149.00	139.00	

Guinea_pig	124.00	24.00	114.00	131.00	209.00	25.00	103.00	25.00	140.00

TLR9

Nile_tilapia_B-actin									
Nile_tilapia	774.00								
Human	706.00	628.00							
Mouse	695.00	633.00	243.00						
Zebra_fish	711.00	511.00	600.00	609.00					
Puffer_fish	735.00	382.00	626.00	623.00	543.00				
Atlantic_salmon	709.00	442.00	607.00	603.00	472.00	457.00			
Common_carp	730.00	491.00	608.00	606.00	222.00	521.00	481.00		
Rainbow_trout	704.00	436.00	603.00	599.00	471.00	452.00	31.00	478.00	
Chimpanzee	708.00	628.00	6.00	247.00	603.00	628.00	609.00	611.00	605.00

Appendix 5 Statistical analysis

ANOVA analysis table of macrophages-like cell stimulated with upPGN compared between control cell, 6 hours and 12 hours after stimulation

Gene	ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
TNF-α	Treatment (between columns)	3.181E+13	2	1.591E+13	F (2, 3) = 0.9723	P = 0.4726
	Residual (within columns)	4.908E+13	3	1.636E+13		
	Total	8.089E+13	5			
IL-1β	Treatment (between columns)	22320000	2	11160000	F (2, 3) = 2.492	P = 0.2304
	Residual (within columns)	13440000	3	4480000		
	Total	35760000	5			
IL-6	Treatment (between columns)	1685000000	2	842500000	F (2, 3) = 0.9138	P = 0.4899
	Residual (within columns)	2766000000	3	922000000		
	Total	4451000000	5			
IL-10	Treatment (between columns)	8.414E+12	2	4.207E+12	F (2, 3) = 1.007	P = 0.4627
	Residual (within columns)	1.253E+13	3	4.176E+12		
	Total	2.094E+13	5			
TGF-β	Treatment (between columns)	9.084E+11	2	4.542E+11	F (2, 3) = 0.5154	P = 0.6421
	Residual (within columns)	2.644E+12	3	8.813E+11		
	Total	3.552E+12	5			
TLR3	Treatment (between columns)	7308000	2	3654000	F (2, 9) = 1.045	P = 0.3906
	Residual (within columns)	31460000	9	3496000		
	Total	38770000	11			
IRF3	Treatment (between columns)	5.114E+10	2	2.557E+10	F (2, 9) = 8.278	P = 0.0091**
	Residual (within columns)	2.78E+10	9	3.089E+09		
	Total	7.895E+10	11			

Viperin	Treatment (between columns)	14170000	2	7085000	F (2, 9) = 7.760	P = 0.0110*
	Residual (within columns)	8217000	9	912987		
	Total	22390000	11			

A t-test table of genes expression analysis between control cell and 12 hrs. After stimulation with both PGN and poly I:C. Stars above *P*-value denote results significantly different at α=0.05.

Genes	Treatment	n	Mean	SD	t	P-value
TNF-α	Control	6	3.41E+05	1.97E+05	1.448	0.2073
	12 hours	6	2.69E+06	4.11E+06		
IL-1β	Control	6	9.46E+03	1.08E+04	1.923	0.1124
	12 hours	6	1.79E+04	1.84E+04		
IL-6	Control	6	3.19E+03	3.85E+03	3.924	0.0111*
	12 hours	6	4.46E+04	2.52E+04		
IL-10	Control	6	1.53E+05	2.04E+05	0.9061	0.4064
	12 hours	6	9.29E+05	2.11E+06		
TGF-β	Control	6	7.38E+03	4.97E+03	2.946	0.032*
	12 hours	6	4.00E+03	3.94E+03		
IRF3	Control	6	2.80E+04	3.22E+04	4.029	0.01*
	12 hours	6	1.08E+06	2.29E+06		
Viperin	Control	6	2.62E+04	2.25E+04	5.463	0.0028*
	12 hours	6	1.02E+06	1.25E+06		
TLR2	Control	6	3.10E+05	5.07E+05	0.7612	0.4809
	12 hours	6	1.41E+05	1.81E+05		
TLR3	Control	6	2.74E+04	4.31E+04	0.7272	0.4997
	12 hours	6	8.97E+04	1.94E+05		
TLR8	Control	6	2.26E+06	2.24E+06	0.1845	0.8608
	12 hours	6	2.07E+06	2.92E+06		
TLR9	Control	6	4.75E+05	8.29E+05	0.0337	0.9744
	12 hours	6	4.61E+05	6.55E+05		

ANOVA analysis table of cytokines mRNA of Nile tilapia compared between head kidney spleen and liver, *P*<0.05

Gene	ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
TNF-α	Treatment (between columns) Residual (within columns) Total	5855000 38420000 44270000	2 15 17	2928000 2561000	F (2, 15) = 1.143	P = 0.3451
IL-1β	Treatment (between columns) Residual (within columns) Total	67790000 5.08E+08 5.76E+08	2 15 17	3.4E+07 3.4E+07	F (2, 15) = 1.000	P = 0.3911
IL-6	Treatment (between columns) Residual (within columns) Total	6743000 50570000 57310000	2 15 17	3371000 3371000	F (2, 15) = 1.000	P = 0.3911
IL-10	Treatment (between columns) Residual (within columns) Total	1.78E+09 2.07E+10 2.25E+10	2 15 17	8.9E+08 1.4E+09	F (2, 15) = 0.6433	P = 0.5394
TGF-β	Treatment (between columns) Residual (within columns) Total	1.23E+10 1.25E+11 1.37E+11	2 15 17	6.1E+09 8.3E+09	F (2, 15) = 0.7380	P = 0.4947

ANOVA analysis table of cytokines mRNA of Mozambique tilapia compared between head kidney spleen and liver, *P*<0.05

Gene	ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
TNF-α	Treatment (between columns) Residual (within columns) Total	6.045E+11 1.656E+11 7.701E+11	2 15 17	3.02E+11 1.1E+10	F (2, 15) = 27.37	P < 0.0001***
IL-1β	Treatment (between columns) Residual (within columns) Total	6.501E+11 3.023E+11 9.525E+11	2 15 17	3.25E+11 2.02E+10	F (2, 15) = 16.13	P = 0.0002***

IL-6	Treatment (between columns) Residual (within columns) Total	2926000 3486000 6412000	2 15 17	1463000 232417	F (2, 15) = 6.294	P = 0.0104***
IL-10	Treatment (between columns) Residual (within columns) Total	8.792E+12 6.413E+12 1.521E+13	2 15 17	4.4E+12 4.28E+11	F (2, 15) = 10.28	P = 0.0015***
TGF-β	Treatment (between columns) Residual (within columns) Total	5.201E+12 8.509E+12 1.371E+13	2 15 17	2.6E+12 5.67E+11	F (2, 15) = 4.584	P = 0.0279*

A t-test table of antiviral genes expression analysis between spleen of Nile tilapia and Mozambique tilapia *P*<0.05

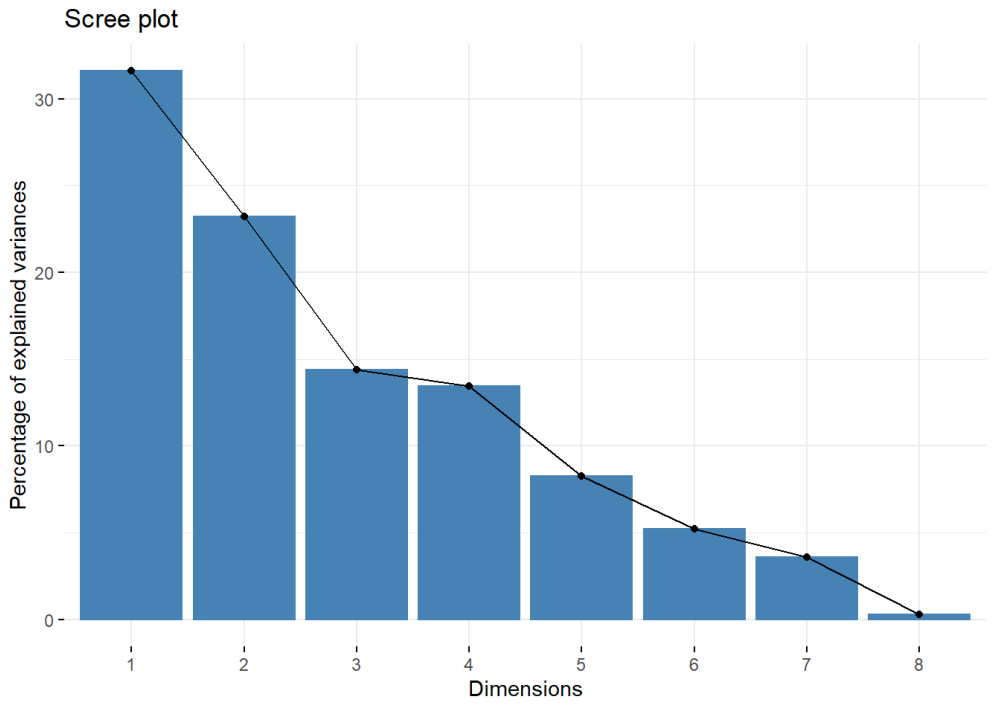
Gene	Treatment	n	Mean	SD	t	P value
TLR3	Nile tilapia Mozambique	6 6	609 2460	831.6 4550	0.952	0.3746
IRF3	Nile tilapia Mozambique	6 6	2018 33283	3105 60862	4.482	0.0021**
Viperin	Nile tilapia Mozambique	6 6	687 13852	677.3 22121	2.815	0.0227*

A t-test table of genes expression analysis between Nile tilapia and red hybrid tilapia sampling from Thailand *P*<0.05

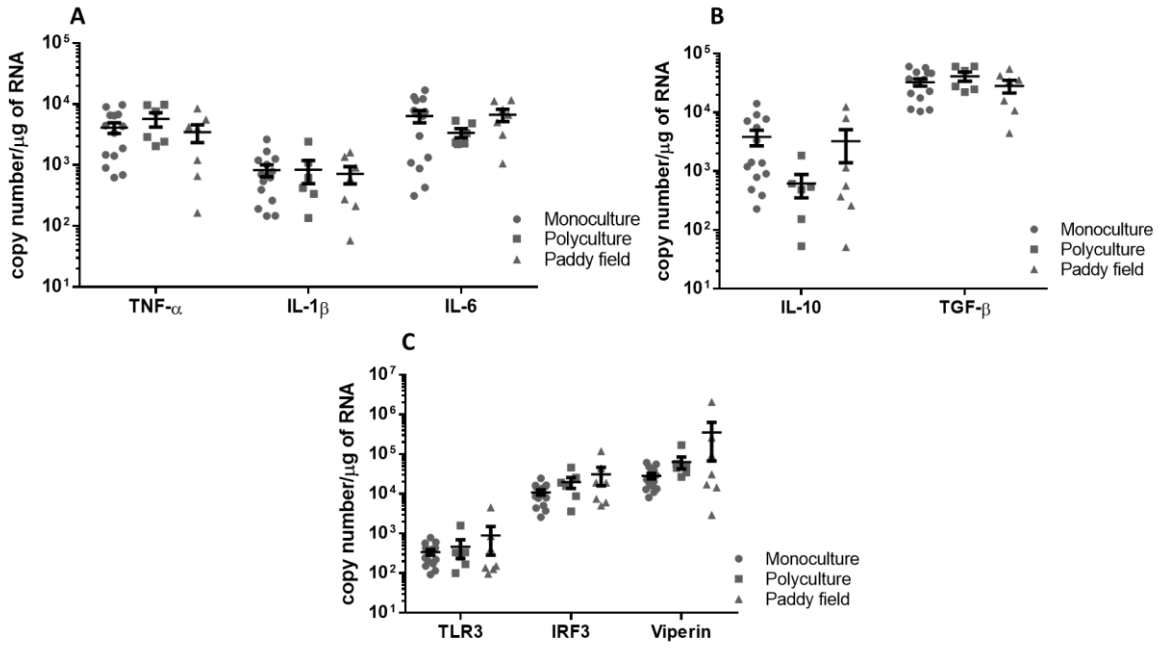
Gene	species	n	Mean	SD	t	P-value
TNF-α	Hybrid tilapia Nile tilapia	25 27	401.3 129.6	394.2 96.5	3.473	0.0011***
IL-1β	Hybrid tilapia Nile tilapia	25 27	173.7 24	140.6 20.51	5.472	<0.0001***
IL-6	Hybrid tilapia Nile tilapia	25 27	96.17 174.4	254.4 136.4	1.396	0.169
IL-10	Hybrid tilapia Nile tilapia	25 27	105.3 89.37	332.1 122	0.2335	0.8164
TGF-β	Hybrid tilapia Nile tilapia	25 27	1917 1008	1346 534.3	3.246	0.0021**

TLR3	Hybrid tilapia	25	92.85	167.1	2.379	0.0212*
	Nile tilapia	27	15.42	25.93		
IRF3	Hybrid tilapia	25	1314	1557	2.336	0.0236*
	Nile tilapia	27	545.8	681.2		
Viperin	Hybrid tilapia	25	34759	151481	1.066	0.2916
	Nile tilapia	27	3612	11701		

A screen plot and principle component data of PCA analysis



	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Standard deviation	1.5909	1.3626	1.0733	1.0373	0.81266	0.64449	0.53501	0.14901
Proportion of Variance	0.3164	0.2321	0.144	0.1345	0.08255	0.05192	0.03578	0.00278
Cumulative Proportion	0.3164	0.5485	0.6925	0.827	0.90952	0.96144	0.99722	1



A comparison of mRNA transcription of innate immunity genes of Nile tilapia among culture condition. Monoculture (n=14), Polyculture (n=6) and Paddy field (n=7). ANOVA and multiple comparison was tested with a level of significance; *P*<0.05. Stars above bar denotes results significantly different. A: pro-inflammatory cytokines B: anti-inflammatory cytokines

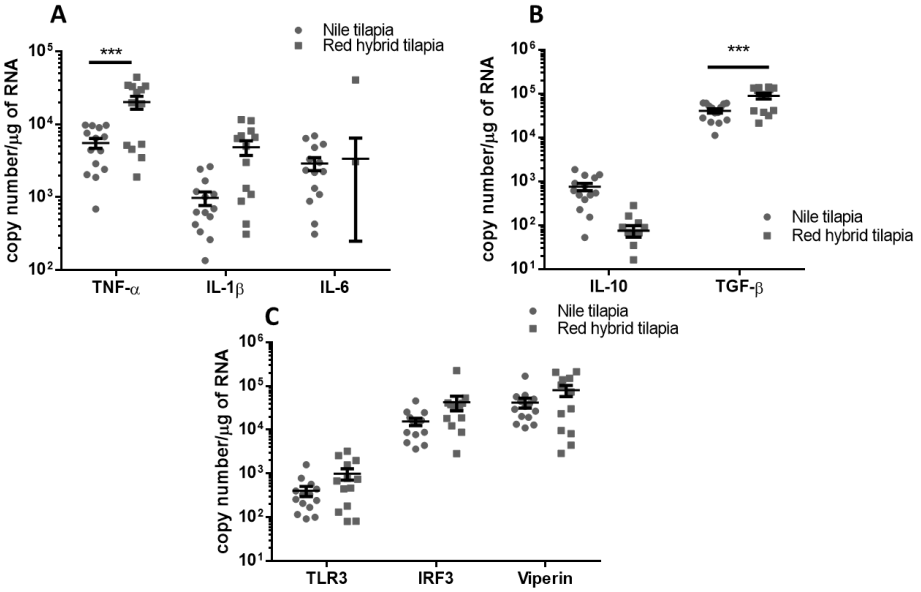
ANOVA analysis table of genes expression of Nile tilapia compared between different culture conditions; monoculture, polyculture, and paddy field, *P*<0.05

Gene	ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
TNF-α	Treatment (between columns)	17600000	2	8800000	F (2, 24) = 0.8417	P = 0.4433
	Residual (within columns)	2.51E+08	24	10450000		
	Total	2.69E+08	26			
IL-1β	Treatment (between columns)	67162	2	33581	F (2, 24) = 0.06680	P = 0.9356
	Residual (within columns)	12070000	24	502725		
	Total	12130000	26			
IL-6	Treatment (between columns)	45670000	2	22840000	F (2, 24) = 1.116	P = 0.3439
	Residual (within columns)	4.91E+08	24	20460000		
	Total	5.37E+08	26			

IL-10	Treatment (between columns) Residual (within columns) Total	44560000 3.85E+08 4.29E+08	2 24 26	22280000 16030000	F (2, 24) = 1.390	P = 0.2685
TGF-β	Treatment (between columns) Residual (within columns) Total	5.57E+08 7.67E+09 8.23E+09	2 24 26	2.79E+08 3.2E+08	F (2, 24) = 0.8715	P = 0.4312
TLR3	Treatment (between columns) Residual (within columns) Total	1472000 17920000 19390000	2 24 26	735983 746576	F (2, 24) = 0.9858	P = 0.3877
IRF3	Treatment (between columns) Residual (within columns) Total	1.94E+09 1.14E+10 1.34E+10	2 24 26	9.7E+08 4.77E+08	F (2, 24) = 2.034	P = 0.1527
Viperin	Treatment (between columns) Residual (within columns) Total	5.15E+11 3.43E+12 3.95E+12	2 24 26	2.58E+11 1.43E+11	F (2, 24) = 1.802	P = 0.1866

ANOVA analysis table of genes expression of red hybrid tilapia compared between different culture conditions; monoculture, polyculture, and paddy field, *P*<0.05

Gene	ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
TNF-α	Treatment (between columns)	2.23E+09	2	1.12E+09	F (2, 22) = 12.86	P = 0.0002***
	Residual (within columns)	1.91E+09	22	86680000		
	Total	4.14E+09	24			
IL-1β	Treatment (between columns)	2.89E+08	2	1.45E+08	F (2, 22) = 13.43	P = 0.0002***
	Residual (within columns)	2.37E+08	22	10760000		
	Total	5.26E+08	24			
IL-6	Treatment (between columns)	63160000	2	31580000	F (2, 22) = 0.4186	P = 0.6631
	Residual (within columns)	1.66E+09	22	75440000		
	Total	1.72E+09	24			
IL-10	Treatment (between columns)	8.85E+08	2	4.43E+08	F (2, 22) = 4.750	P = 0.0193***
	Residual (within columns)	2.05E+09	22	93150000		
	Total	2.93E+09	24			
TGF-β	Treatment (between columns)	2.82E+10	2	1.41E+10	F (2, 22) = 15.56	P < 0.0001***
	Residual (within columns)	2E+10	22	9.08E+08		
	Total	4.82E+10	24			
TLR3	Treatment (between columns)	4.27E+08	2	2.14E+08	F (2, 22) = 14.87	P < 0.0001***
	Residual (within columns)	3.16E+08	22	14360000		
	Total	7.43E+08	24			
IRF3	Treatment (between columns)	1.45E+10	2	7.25E+09	F (2, 22) = 3.188	P = 0.0608
	Residual (within columns)	5.01E+10	22	2.28E+09		
	Total	6.46E+10	24			
Viperin	Treatment (between columns)	7.15E+10	2	3.57E+10	F (2, 22) = 1.640	P = 0.2168
	Residual (within columns)	4.79E+11	22	2.18E+10		
	Total	5.51E+11	24			



A comparisons between the different strain of tilapia to innate immune response using two-way ANOVA (α=0.05). Stars above bar denotes results significantly different. Nile tilapia (n=14) and red hybrid tilapia (n=13) were cultured in earth pond.

ANOVA analysis table, a comparison between Nile tilapia and red hybrid tilapia cultured in earth pond (α=0.05).

Gene	Species	ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
TNF-α	Nile tilapia	Treatment (between columns)	7.61E+03	2	3.81E+03	F (2, 24) = 0.3894	P = 0.6817
		Residual (within columns)	2.35E+05	24	9.77E+03		
		Total	2.42E+05	26			
	Hybrid tilapia	Treatment (between columns)	2.01E+06	2	1.01E+06	F (2, 22) = 12.86	P = 0.0002***
		Residual (within columns)	1.72E+06	22	7.82E+04		
		Total	3.73E+06	24			
IL-1β	Nile tilapia	Treatment (between columns)	6.06E+01	2	3.03E+01	F (2, 24) = 0.06680	P = 0.9356
		Residual (within columns)	1.09E+04	24	4.53E+02		
		Total	1.09E+04	26			

	Hybrid tilapia	Treatment (between columns)	2.61E+05	2	1.30E+05	F (2, 22) = 13.43	P = 0.0002***
		Residual (within columns)	2.14E+05	22	9.71E+03		
		Total	4.74E+05	24			
IL-6	Nile tilapia	Treatment (between columns)	4.12E+04	2	2.06E+04	F (2, 24) = 1.116	P = 0.3439
		Residual (within columns)	4.43E+05	24	1.84E+04		
		Total	4.84E+05	26			
	Hybrid tilapia	Treatment (between columns)	5.70E+04	2	2.85E+04	F (2, 22) = 0.4186	P = 0.6631
		Residual (within columns)	1.50E+06	22	6.80E+04		
		Total	1.55E+06	24			
IL-10	Nile tilapia	Treatment (between columns)	4.02E+04	2	2.01E+04	F (2, 24) = 1.390	P = 0.2685
		Residual (within columns)	3.47E+05	24	1.45E+04		
		Total	3.87E+05	26			
	Hybrid tilapia	Treatment (between columns)	7.98E+05	2	3.99E+05	F (2, 22) = 4.750	P = 0.0193**
		Residual (within columns)	1.85E+06	22	8.40E+04		
		Total	2.65E+06	24			
TGF-β	Nile tilapia	Treatment (between columns)	5.03E+05	2	2.51E+05	F (2, 24) = 0.8715	P = 0.4312
		Residual (within columns)	6.92E+06	24	2.88E+05		
		Total	7.42E+06	26			
	Hybrid tilapia	Treatment (between columns)	2.55E+07	2	1.27E+07	F (2, 22) = 15.56	P < 0.0001***
		Residual (within columns)	1.80E+07	22	8.19E+05		
		Total	4.35E+07	24			
TLR3	Nile tilapia	Treatment (between columns)	1.33E+03	2	6.64E+02	F (2, 24) = 0.9858	P = 0.3877
		Residual (within columns)	1.62E+04	24	6.73E+02		
		Total	1.75E+04	26			
	Hybrid tilapia	Treatment (between columns)	3.85E+05	2	1.93E+05	F (2, 22) = 14.87	P < 0.0001***
		Residual (within columns)	2.85E+05	22	1.30E+04		

		Total	6.70E+05	24			
IRF3	Nile tilapia	Treatment (between columns)	1.75E+06	2	8.74E+05	F (2, 24) = 2.034	P = 0.1527
		Residual (within columns)	1.03E+07	24	4.30E+05		
		Total	1.21E+07	26			
	Hybrid tilapia	Treatment (between columns)	1.31E+07	2	6.54E+06	F (2, 22) = 3.188	P = 0.0608
		Residual (within columns)	4.51E+07	22	2.05E+06		
		Total	5.82E+07	24			
Viperin	Nile tilapia	Treatment (between columns)	4.65E+08	2	2.32E+08	F (2, 24) = 1.802	P = 0.1866
		Residual (within columns)	3.10E+09	24	1.29E+08		
		Total	3.56E+09	26			
	Hybrid tilapia	Treatment (between columns)	7.15E+10	2	3.57E+10	F (2, 22) = 1.640	P = 0.2168
		Residual (within columns)	4.79E+11	22	2.18E+10		
		Total	5.51E+11	24			

A t-test table of genes expression analysis between Monoculture and Polyculture in earth pond of Nile tilapia, *P*<0.05

Gene	Treatment	n	Mean	SD	t	P value
TNF-α	monoculture	8	5428	3201	0.1664	0.8706
	polyculture	6	5735	3685		
IL-1β	monoculture	8	1083	766	0.0524	0.5842
	polyculture	6	840.7	844		
TGF-β	monoculture	8	39873	17201	0.1525	0.8813
	polyculture	6	41325	18223		
IRF3	monoculture	8	12225	6955	1.273	0.2272
	polyculture	6	19770	14881		

A t-test table of genes expression analysis between Monoculture and Polyculture in earth pond of red hybrid tilapia, *P*<0.05

Gene	Treatment	n	Mean	SD	t	P value
TNF-α	monoculture	7	12145	13863	2.603	0.0245*
	polyculture	6	29704	9629		
IL-1β	monoculture	7	1927	2177	4.76	0.0006***
	polyculture	6	8292	2650		
TGF-β	monoculture	7	61819	47548	2.738	0.0193*
	polyculture	6	119270	20428		
IRF3	monoculture	7	18706	12508	1.82	0.0961
	polyculture	6	71004	75379		