Comparative Expression Analysis of Toll-Like Receptor Protein in Brain, Gill and Liver of Healthy and Diseased Empurau (*Tor tambroides*)

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Bachelor of Science with Honours
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Comparative Expression Analysis of Toll-Like Receptor Protein in Brain, Gill and Liver of Healthy and Diseased Empurau (*Tor tambroides*)

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A thesis submitted in partial fulfilment of the Requirement of The Degree Bachelor of Science with Honours

(Resource Biotechnology)

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Programme of Resource Biotechnology
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2022

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ABSTRACT

The toll-like receptor proteins are pattern recognition receptors (PRRs) that trigger the innate immune systems to detect and recognize the conserved pathogen-associated molecular patterns (PAMPs) to engage the early immunological recognition of various types of pathogens. Various pathogens can cause infections (e.g., dropsy disease) in many aquaculture species, leading to significant economic losses for the producers. Infectious dropsy is one of the bacterial hemorrhagic septicemia diseases characterized by the abnormal accumulation of water or other fluids in the whole body, especially in the abdomen or localized in different organs of the fish. Although *Tor tambroides* is one of the famous Malaysian mahseers, yet there is not enough information on their TLR repertoire to fully comprehend the underlying molecular regulation of pathogens recognition. This research aims to isolate the total RNA and to compare the toll-like receptor gene expressions on different organs of healthy and diseased *Tor tambroides*. *TLR1*, *TLR3*, *TLR5*, *TLR7*, and *TLR9* have been selected from the whole *Tor tambroides* genome for primer design after being blasted with the transcriptomic data of *Tor tambra*. Gene expression analysis was performed via semi-quantitative RT-PCR using the cDNA of three isolated organs (brain, gill, and liver) from healthy and diseased *Tor tambroides*, respectively. Expression was detected in the diseased liver for *TLR1*, *TLR3*, *TLR5*, and *TLR7* genes might suggest the role of TLRs in recognition of the invasion of pathogens. The pathogen focused on this research is *Pseudomonas fluorescens*. Identifying of the TLRs and their roles may contribute to a better understanding of the disease resistance mechanisms in *Tor tambroides*, as well as a new insight for drug design to regulate the immune response.

Key words: toll-like receptors, innate immune system, *Tor tambroides*, dropsy disease, gene expression

ABSTRAK

Protein reseptor seperti tol ialah reseptor pengecaman corak (PRR) yang mencetuskan sistem imun semula jadi untuk mengesan dan mengenali corak molekul berkaitan patogen (PAMP) yang dipelihara untuk melibatkan pengecaman imunologi awal pelbagai jenis patogen. Pelbagai patogen boleh menyebabkan jangkitan (cth., penyakit dropsy) dalam banyak spesies akuakultur, yang membawa kepada kerugian ekonomi yang ketara bagi pengeluar. Infectious dropsy adalah salah satu penyakit septikemia hemoragik bakteria yang dicirikan oleh pengumpulan air atau cecair lain yang tidak normal di seluruh badan, terutamanya di dalam perut atau disetempat di organ yang berbeza ikan. Walaupun Tor tambroides adalah salah satu mahseer Malaysia yang popular, namun tidak ada maklumat yang mencukupi tentang himpunan TLR mereka untuk memahami sepenuhnya peraturan molekul yang mendasari pengiktirafan patogen untuk pengukuran yang berkesan. Matlamat penyelidikan ini adalah untuk mengasingkan jumlah RNA, dan untuk membandingkan ekspresi gen reseptor seperti tol pada organ berbeza Tor tambroides yang sihat dan berpenyakit. TLR1, TLR3, TLR5, TLR7, dan TLR9 telah dipilih daripada keseluruhan genom Tor tambroides untuk reka bentuk primer selepas diletupkan dengan data transkriptom Tor tambra. Analisis ekspresi gen dilakukan melalui RT-PCR separa kuantitatif menggunakan cDNA tiga organ terpencil (otak, insang, hati) daripada Tor tambroides yang sihat dan berpenyakit. Ekspresi tinggi dikesan dalam hati yang berpenyakit untuk TLR1, TLR3, TLR5, dan TLR7 yang mungkin mencadangkan bahawa peranan TLR dalam mengiktiraf pencerobohan patogen. Patogen yang difokuskan pada penyelidikan ini ialah Pseudomonas fluorescens. Mengenal pasti TLR dan peranan mereka mungkin menyumbang kepada pemahaman yang lebih baik tentang mekanisme rintangan penyakit dalam Tor tambroides, serta wawasan baharu untuk reka bentuk ubat untuk mengawal tindak balas imun.

***Kata kunci**: Protein reseptor seperti tol, sistem imun semula jadi, Tor tambroides, penyakit dropsy*, ekspresi gen

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LIST OF ABBREVIATIONS

AGE	Agarose Gel Electrophoresis
APC	Antigen-Presenting Cells
AP-1	Activator Protein 1
BLASTn	Nucleotide Basic Local Alignment Search Tool
CFU	Colony Forming Unit
CLRs	C-Type Lectin Receptors
CpG ODN	CpG Oligodeoxynucleotides
DAMPs	Damage-Associated Molecular Patterns
ddH_2O	Double-Distilled Water
dsRNA	Double-Stranded RNA
ER	Endoplasmic Reticulum
EtBr	Ethidium Bromide
ExPASy	Expert Protein Analysis System
FAO	Food and Agriculture Organization
GCRV	Grass Carp Reovirus
IDT	Integrated DNA Technologies
IFRPC	Indigenous Fisheries Research and Production Centre
IL-1	Interleukin-1
IL-6	Interleukin-6
IUCN	International Union for Conservation of Nature
LB	Luria Broth
LPS	Lipopolysaccharides
LRRs	Leucine-Rich Repeats
LTA	Lipoteichoic Acid

MEGA	Molecular Evolutionary Genetics Analysis
MHC	Major Histocompatibility Complex
mTLR5	Membrane Toll-Like Receptor 5
MyD88	Myeloid Differentiation Primary Response Protein 88
NF-$_{K}$B	Nuclear Factor Kappa B
NLRs	Nucleotide-Binding Oligomerization Domain-Like Receptors
ORFs	Open Reading Frames
PAMPs	Pathogen-Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PG	Peptidoglycan
PRRs	Pattern Recognition Receptors
PRP/R	Pattern Recognition Proteins Or Receptors
RLRs	RIG-1-Like Receptors
SHRV	Snakehead Rhabdovirus
SNPs	Single Nucleotide Polymorphisms
sTLR5	Soluble Toll-Like Receptor 5
ssRNA	Single-Stranded RNA
TBE	Tris-Borate
TEC	Tata Electric Company
TIR	Toll-Interleukin-1 Receptor
TLRs	Toll-Like Receptors
TNF-α	Tumour Necrosis Factor Alpha

CHAPTER 1: INTRODUCTION

1.1 Study Background

The Malaysian Fisheries Sector, which includes marine capture fishery, inland fisheries, and aquaculture, have become one of the fastest-developing sectors that give and contribute to the economic prosperity in Malaysia. Fish and other aquatic products are recognised as one of the healthiest foods on the planet. These foods are high and rich in protein contents, as well as contain many essential micronutrients such as iron, zinc and calcium. Due to these reasons, the fisheries sectors have played a significant role in global, national and regional food security and nutrition plans, assisting in transforming food systems and eradicating malnutrition and hunger (Food and Agriculture Organization (FAO), 2020). In Malaysia, fishery and aquaculture sectors have provided a broad range of employment opportunities to more than 140,000 people in 2017, which helped them support their daily livelihood (Waiho et al., 2020). According to Food and Agriculture Organization (FAO) (2020), the total fish production is expected to increase from 179 million tonnes in 2018 to 204 million tonnes in 2030. In 2030, aquaculture production is expected to reach 109 million tonnes, up 32% (26 million tonnes) from 2018.

Malaysian mahseer, *Tor tambroides*, commonly named Empurau or Kelah in Malaysia, Jurung in Indonesia (Jaafar et al., 2021), is one of the fish members of the family Cyprinidae that is widespread species targeted by fisheries and aquaculture mainly for human consumption (Kottelat et al., 2018). Since the Empurau possesses an excellent texture and taste, thus is being characterized as an expansive edible freshwater fish, and the cost typically ranges from RM800 to RM1000 per kilogram (Bernama, 2021). As true mahseer (*Tor spp*.), it can be found within fast-flowing rivers with rocky bottoms (Shreshtha, 1997). Like other *Tor spp*., *Tor tambroides* are threatened by environmental degradation in the wild, causing an elevating decline in their population size in recent years (Ingram et al., 2005). Deforestation and exploitation, logging, overfishing, as well as the modification of river morphology anthropogenically have interrupted

and reduced the water flow within their habitat. Consequently, these activities not only imperil the population size of Empurau but it is also brought severe negative impacts on the aquatic environments. This condition might discourage or give rise to a poor consequences on the sales of fisheries.

Apart from environmental factors and anthropogenic activities, dropsy disease is also a possible factor contributing to the declining Empurau population. Dropsy disease, also called ascites, is a medical condition that causes the soft tissue in the coelomic cavity of the organism to swell and distend due to accumulation of water and other fluids (Densmore, 2019). Infectious dropsy poses one of the most significant threats to the economic losses in the aquaculture sector (Meyer, 1991). The maintenance of a massive number of fishes crowded together in a narrow area can provide a conducive environment for the development and spread of infectious disease (Alaliyat & Yndestad, 2015). In this unnatural crowded living area, fishes are stressed and more susceptible to infectious disease. According to Haryo & Nurhidayati (2020), a study conducted on *Cyprinus carpio* stated that the bacterial, viral, or other pathogenic infections associated with the variation of living factors such as temperature, nutrition, and water environment could cause severe dropsy infection in this fish species and has a higher possibility to transmit to other fishes.

According to Kawai & Akira (2010), toll-like receptors, abbreviated as TLRs, are germ-line encoded pathogen recognition receptor that allow the innate immune system of an organism to distinguish between pathogens by recognising the pathogen-associated molecular patterns. There is currently a lack of study to fill up the knowledge gap in understanding dropsy disease in Empurau. In this study, the presence of the toll-like receptors (TLRs), including *TLR1*, *TLR3*, *TLR5*, *TLR7*, and *TLR9* in *Tor tambroides* was determined which postulate that these genes will involve in triggering the innate immune responses and serve as fundamental for understanding an of the dropsy disease in *Tor tambroides*.

1.2 OBJECTIVES

The objectives of this research study are:

1. To isolate the total RNA from both healthy and diseased *Tor tambroides*.
2. To compare the toll-like receptor genes expression on different organs of healthy and diseased *Tor tambroides*.

CHAPTER 2: LITERATURE REVIEW

2.1 *Tor tambroides*

The Malaysian mahseer, *Tor tambroides*, also commonly known as Empurau or Kelah in Malaysia, Jurung in Indonesia (Jaafar et al., 2021), is one of the most valuable freshwater fishes found in Southeast Asia (Lau et al., 2021). It is an ornamental, sport, and edible freshwater fish found in Malaysia. Empurau is one of the fish members of the family Cyprinidae, sharing a similar biogeographical distribution with *T. tambra* and *T. douronensis* across Malaysia and Indonesia. In 2005, local commercial farms started initiating mahseer cultures by catching the wild mahseer, giving rise to the first mahseer farming in Malaysia. Successfully induced captive breeding techniques also showed an exponentially increase in aquaculture production. Due to this reason, Empurau possesses an excellent texture and attractive features and taste, thus being touted as the costliest cultured fish with prices fetching as high as USD200/kg in the Malaysian market (Ishak, 2020). A unique cultural property with the continuous economic influences of Empurau has a significant impact on local aquaculture industries and is highly prized by the local communities, especially in Sarawak (De Silva et al., 2004).

According to Shreshtha (1997), the native habitat of *Tor tambroides* is fast-flowing water with rocky bottoms. However, environmental degradation such as river pollution, logging and deforestation, with the huge increment of uncontrolled human activities such as uncontrolled fish harvesting by the local or illegal fish poaches, make these habitats unsustainable for the survival of wild mahseer species (Walton et al., 2017). These practices had affected and led to the rapid reduction of the population size of Empurau, particularly in Peninsular Malaysia. Currently, a breeding programme has also been established by the Department of Agriculture to develop the cultural techniques and artificial propagation for Empurau, which is essential for aquaculture and conservation purposes.

2.1.1 Morphology of *Tor tambroides*

Tor tambroides possess some unique body morphologies, which were first described by Bleeker (1854) as shown in Figure 2.1. According to Bleeker (1854), *Tor tambroides* possess a total of four dorsal spines, eight dorsal soft rays, three anal spines, five anal soft rays and 39 to 41 vertebrae. It has a lengthy median lobe that reaches an imaginary line between the corners of the mouth on the lower lip. Besides, its upper lip was rolled backwards and upwards with an upwardly extending median lobe. The upward projecting median lobe with a ventral view of the eyes is considered a critical physical morphology as these assist the fishes to withstand the living streams. In adult fish, there was the absence of a dark longitudinal stripe along the side in adults. Its fins will also turn from yellow (growth stage) into blackish colour (adult stage). Upon maturity, it can grow up to the length range between 61 cm to 74.9 cm and can reach a maximum length 1 meter (Bleeker, 1854). In brief, a streamlined cylindrical body with a muscular tail and hypertrophied lips allow them to withstand, live and swim in a fast-flowing water living environment.

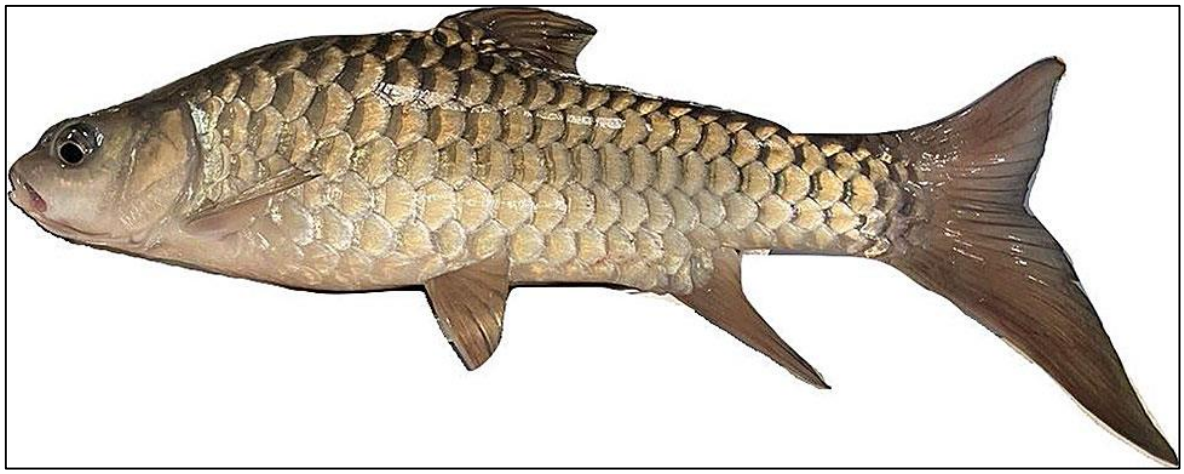

Figure 2.1: Image of Malaysian mahseer, *Tor tambroides* (Adapted from Lau et al., 2021)

2.1.2 Captive breeding of *Tor tambroides*

According to Ralls & Ballou (2013), captive breeding is often known as a conservation breeding project that allows the zoo, wildlife reserves, and conservation facilities to exhibit a wide range of species without capturing new individuals from the wild. The primary purpose

of establishing captive breeding is to maintain and preserve the species that has been reduced to a very tiny number of individuals, including Empurau. The early hatchery production of mahseer juveniles was produced from hand-stripping wild-caught mature spawners during the breeding seasons, with or without hypophysation (Ogale, 2002). Currently, this method has been replaced by the use of pond-reared broodstock (Gurung et al., 2002; Ingram et al., 2005). The breeding approaches and artificial propagation have become available for many mahseer species, such as *T. khudree*, *T. putitora*, *T. tor*, and *T. tambroides*.

In the 1970s, the Tata Electric Company (TEC) at Lonavla, Maharashtra was the first to conduct captive breeding in mahseer species for sake of conservation and stock enhancement. Since then, this practice has been gradually spread to Nepal, Bangladesh, as well as Malaysia. In East Malaysia, *Tor tambroides* are being threatened, jeopardised in the wild and showing the trend of a decline in abundance and distribution due to natural habitat degradation and daily anthropogenic activities such as overfishing. However, there is a lack of the stronger supportive statement from the International Union for Conservation of Nature (IUCN) stating or listing that Empurau is an extinct species (Pinder et al., 2019).

The drastic reduction of the Empurau has raised awareness among related societies or authorities such as Department of Fisheries Malaysia regarding the importance of appropriate management and conservation of Empurau (Esa et al., 2008). Consequently, the Department of Agriculture Sarawak has embarked on the research and established a breeding programme on the artificial propagation or culture methods for the aquaculture and conservation goals of Empurau. A unique facility, the Indigenous Fisheries Research and Production Centre (IFRPC) located in Tarat, Serian, has been set up for these goals (Ingram et al., 2007; De Silva et al., 2004).

In order to succeed in breeding, breeders must prior understand and be concerned about several biological factors such as behavioural, ecological, genetic, and ethical issues. In the

research conducted by Lee et al. (2014), captive breeding of Empurau for market purposes in aquaculture ponds showed that an inappropriate water living environment and unsuitable feed could result in a slow production of this species. Thus, all the breeders should manage the bacteriological quality of water properly as the farmed fish diseases can spread likely. Other factors such as organic matter, nutrient salts and dissolved oxygen are also crucial factors that can affect the optimal growth of Empurau in captive breeding.

2.2 Dropsy disease

Disease-related issues commonly arise in aquaculture systems as a result of intensive fish practices, which include the poor biosecurity measurement by the breeder and excessive fish stoking density cultivation that frequently results in low water quality, which eventually gives rise to fish mortality (Liu et al., 2018). Besides, the disease outbreaks are also caused by an uncontrolled transmission from the wild stocks, accidental transfers of diseased stocks between farms, and the inability of the management to recognise the infected fish stocks (Natnan et al., 2021). This might lead to a huge economic loss and could lead to the collapse of the industry of aquaculture if the preventive measures are not taken or planned appropriately.

According to Shome et al. (2016), dropsy disease is a medical condition characterized by the abnormal accumulation of water or other fluids in whole body, especially in the abdomen or localized in different organs of the fish. Dropsy disease is one of the bacterial hemorrhagic septicemia diseases that has been highlighted by many researchers (Aly & Ismail, 2016) in a broad range of cultured fish such as common carps (*Cyprinus carpio*) (Aly & Ismail, 2016), Indian carps (*Labeo rohita*, *Catla catla* and *Cirrhinus mrigala*) (Dash et al., 2008), and silver carp (*Hypopthalmichthys molitrix*) (Lopamudra & Nayak, 2020), as well as the *Tor tambroides* (Lau et al., 2021).

Dropsy disease can be marked and indicated easily from the fish's physical characteristics. According to Aly and Ismail (2016), the typical clinical symptoms of dropsy disease include ascites, coelomic distension, haemorrhagic lesions found on eyes, fins, skin and tail as shown in Figure 2.2. Besides, the fish also have sluggish balancing and difficulty moving, swimming, feeding, and breathing due to a swollen stomach (Haryo & Nurhidayati, 2020). The untreated dropsy can be fatal; early and immediate treatment can still give a recovery chance to the fish.

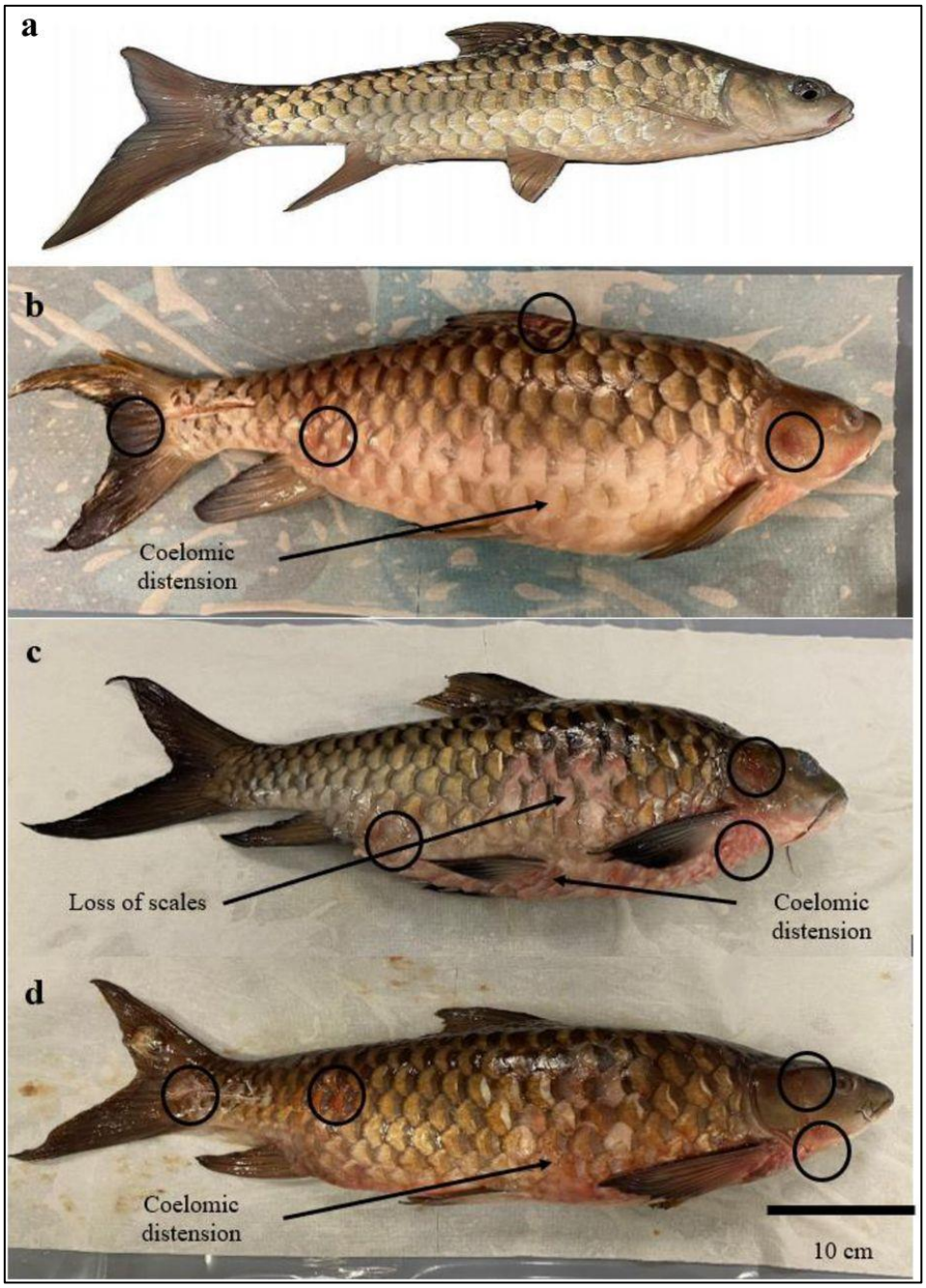

Figure 2.2: Healthy and diseased *Tor tambroides* with the clinical symptoms labelled, including coelomic distension and loss of scales pointed out by the arrow and haemorrhagic lesions circled on tail, eyes, skin, and gills (Adapted from Lau et al., 2021)

Dropsy may be caused by several etiological agents, either infectious or non-infectious. As a bacterial hemorrhagic septicemia disease, it is caused by *Pseudomonas fluorescens* (Aly & Ismail, 2016), which act as a primary pathogen of freshwater fish and an opportunistic species in aquaculture (Shiose et al., 1974). Other research also highlighted that *Aeromonas hydrophila* bacteria may act as a primary pathogen for fish, secondary invaders for hemorrhagic septicemia cases (Kozińska et al., 2002; Soni et al., 2021). Apart from the bacteria infections, numerous other potential etiologies such as viral, parasitic and other pathogenic infections can give rise to the coelomic fluids accumulation as proven from the differential diagnosis conducted by Densmore (2019). Temperature, nutrition, and living environment are also some critical factors that cause the emergence of dropsy in fish. The interaction of these three components causes the fish to become easily stressed, and as a result, the fish's defence becomes weak and readily infected by the dropsy disease. Therefore, some diagnostics methods, including assessing the coelomic fluids via microbial and cytology culture and celiocentesis, are often used in dropsy diagnostic and identify the appropriate management options.

2.3 Immune system and immune response in fish

An immune system is a biological system that protects an organism against diseases by detecting and identifying various types of etiological agents, from parasitic worms to viruses, eradicating the pathogens and suppressing the emergence of tumours (Magnadottir, 2010). The immune system is comprising two major sub-systems: the innate and adaptive immune systems. If pathogens are recognised, the innate immune system will first provide an immediate (non-specific) response (Litman et al., 2005). If the pathogens successfully invade the innate immune system, the second protection which is the adaptive immune system will be activated to improve the recognition of pathogens and engage those pathogens with specificity and immunological memory (Mayer, 2010).

According to Urbinati et al. (2020), fish possess both the innate and adaptive immune systems, both of which present the cell-mediated defence mechanisms and humoral factors. The innate immune system is more prominent than the adaptive immune system (Magnadottir, 2010). It can be divided into three common compartments which include cellular components, humoral parameters, and epithelial or mucosal barrier. As the fishes are frequently immersed or live in the fluids that might contain potentially hazardous agents, the epithelium or mucosal barriers of the skin, gill and alimentary tract are playing an extremely important role as the disease barrier of the fish (Magnadottir, 2010). The humoral parameters are generally expressed in two distinct forms, either or as secreted soluble forms as cell receptors. These include the complement system that involves the alternative, lectin, and classical pathways (Figure 2.3) that are well-developed in fish. These three essential pathways can enhance the phagocytosis by opsonisation of pathogens and activate the adaptive immune response via the classical pathways (Nonaka & Smith, 2000; Boshra et al., 2006).

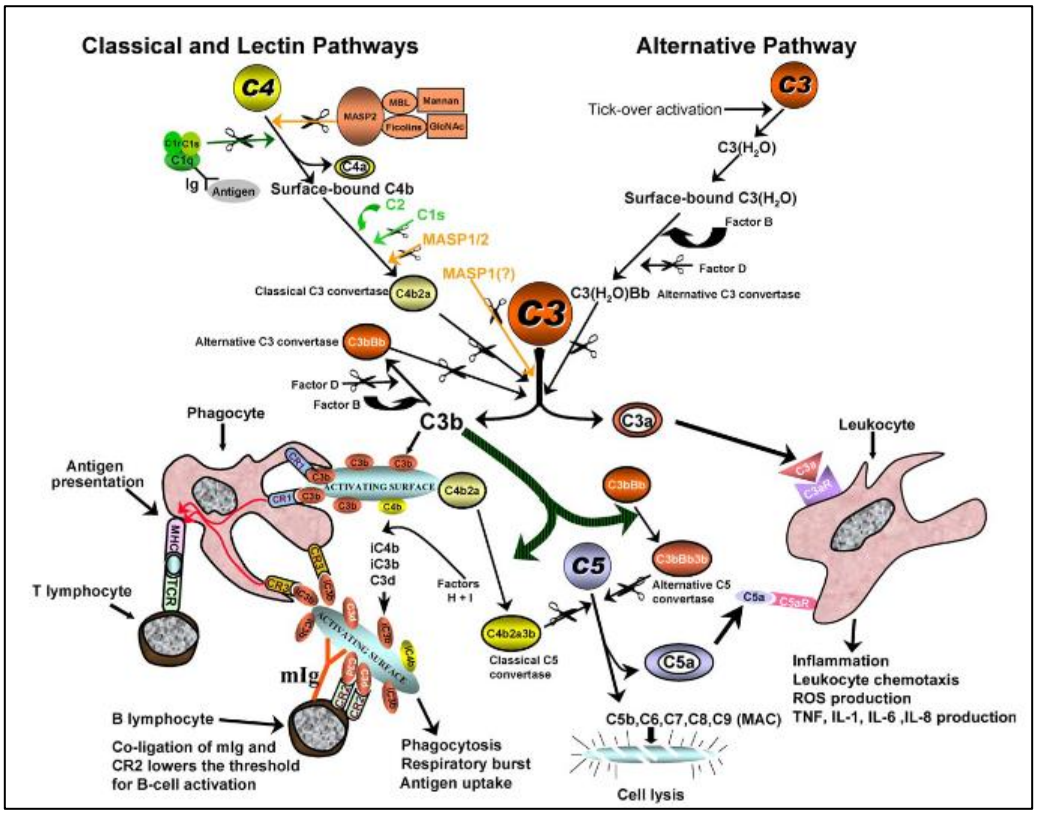

Figure 2.3: Activation of the complement system through the three existing pathways (alternative, lectin, and classical) (Adapted from Boshra et al., 2006)