

Faculty of Resource Science and Technology

Microbial Profiling of Dropsy Diseased Empurau (Tor tambroides)

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Microbial Profiling of Dropsy 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 Faculty of Resource Science and Technology UNIVERSITI MALAYSIA SARAWAK

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Microbial Profiling of Dropsy Diseased Empurau (Tor tambroides)

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ABSTRACT

The highly profitable riverine fish with excellent taste, Tor tambrodies (commonly known as Empurau) is an important fish species in aquaculture business. The study was performed to identify bacterial pathogens from dropsy diseased T. tambroides, compare the isolated bacteria from dropsy and healthy Empurau as well as to assess their sensitivity to antibiotics. Pure culture of bacteria using spread plate techniques, Gram's Staining, KOH string test, and 16s rRNA sequencing were performed to identify the causative agents of the diseased fish. Five types of antibiotics discs namely, Ampicilin (10µg), Tetracycline (30µg), Chloramphenicol (30µg), Clindamycin (2µg) and Morepenem (10µg) were used to test the sensitivity of the isolated bacteria. Three pathogenic bacteria such as Pseudomonas fluorescens, Citrobacter freundii and Aeromonas veronii were identified in the studied diseased Empurau (T. tambroides). Among them, Aeromonas veronii were only identified in the guts of healthy fish but are not identified in dropsy fish guts. P. fluorescens, P. congelans and Citrobacter freundii was found only in diseased Empurau which may perhaps responsible for the Dropsy. The results of the antibiotic sensitivity test showed multiresistances of the identified bacteria to the tested antibiotics. Tetracycline (30µg) and Morepenem (10 µg) was found sensitive to the identified bacteria. Multiple reaction was reported on the use of Ampicilin (10µg) and Chloramphenicol (30µg), but the studied bacteria were resistant to Clindamycin $(2\mu g)$. The results of this study will be helpful to the fish farmers for the management of bacterial diseases in fish. Further research on the biochemical characterization and proper analysis on disease management of capture bred Empurau fish could be done to improve the aquaculture sectors.

Keywords: Empurau (Tor tambroides), dropsy disease, microbial profiling, multi-resistant antibiotics bacteria

ABSTRAK

Ikan sungai yang sangat menguntungkan dengan rasa yang sangat enak, Tor tambrodies (biasanya dikenali sebagai Empurau) ialah spesies ikan yang penting dalam perniagaan akuakultur. Kajian ini dilakukan untuk mengenal pasti patogen bakteria daripada T. tambroides yang mempunyai penyakit dropsy, membandingkan bakteria yang terdapat di dalam isi perut ikan Empurau yang mempunyai dropsy dan Empurau yang sihat, serta menilai sensitiviti mereka terhadap antibiotik. Kultur tulen bakteria menggunakan teknik "spread plate", Pewarnaan Gram, ujian rentetan KOH, dan penjujukan rRNA 16s telah dilakukan untuk mengenal pasti agen penyebab kepada ikan yang berpenyakit. Lima jenis cakera antibiotik iaitu, Ampicilin (10µg), Tetracycline (30µg), Chloramphenicol (30µg), Clindamycin (2µg) dan Morepenem (10µg) telah digunakan untuk menguji sensitiviti bakteria yang telah didapati. Tiga bakteria patogen seperti Pseudomonas fluorescens, Citrobacter freundii dan Aeromonas veronii telah dikenal pasti dalam Empurau berpenyakit yang dikaji (T. tambroides). Aeromonas veronii hanya dikenal pasti dalam isi perut ikan yang sihat tetapi tidak dikenal pasti dalam isi perut ikan yang mempunyai dropsy. P. fluorescens, P. congelans dan Citrobacter freundii hanya ditemui dalam Empurau yang berpenyakit, memberi kemungkinan bahawa ia bertanggungjawab dalam menyebabkan penghasilan penyakit Dropsy. Keputusan ujian sensitiviti antibiotik menunjukkan pelbagai rintangan bakteria yang dikenal pasti terhadap antibiotik yang diuji. Tetracycline (30µg) dan Morepenem (10 µg) didapati sensitif terhadap bakteria yang dikenal pasti. Pelbagai tindak balas telah dilaporkan mengenai penggunaan Ampicilin (10µg) dan Chloramphenicol (30µg), manakala kesemua bakteria yang dikaji adalah kebal terhadap Clindamycin (2µg. Hasil kajian ini akan membantu penternak ikan dalam pengurusan penyakit bakteria pada ikan. Kajian lanjut mengenai pencirian biokimia dan analisis yang betul mengenai pengurusan penyakit ikan Empurau baka tangkapan boleh dilakukan untuk menambah baik sektor akuakultur.

Kata kunci: Empurau (Tor tambroides), penyakit dropsy, profil mikrob, bakteria multiresistant antibiotik

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LIST OF ABBREVIATIONS

AGE	Agarose Gel Electropherosis
BLAST	Basic Local Alignment Search Tool
EDTA	Ethylenediamene tetraacetic acid
IAD	Infectious Abdominal Dropsy
IUCN	International Union for Conservation of Nature
КОН	Potassium Hydroxide
Μ	Molar
MEGA	Molecular Evolutionary Genetics Analysis
mg	Milligram
mL	Millilitre
μL	Microlitre
μΜ	Micrometre
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
nm	Nanometre
OD	Optical Density
PCR	Polymerase Chain Reaction
rDNA	Recombinant DNA
rpm	Revolutions per minute
TBE buffer	Tris-borate-EDTA buffer
TAE buffer	Tris-acetate-EDTA buffer

CHAPTER 1

INTRODUCTION

Bacterial infections in fish are one of the issues affecting the long-term viability of aquaculture output in most regions worldwide (Aly & Ismail, 2016). According to the article, Infectious abdominal dropsy (IAD), often known as dropsy disease, is a bacterial sickness that has been documented by various researchers. Based on Sharpe (2020), dropsy disease is a term used to describe swelling of soft tissue in the body cavity such as abdomen. This disease is common among freshwater aquaculture fish and the fish will appear to have a swollen bellies with other physical condition (Barrington, 2021). Based on the study by Aly and Ismail (2016), bacterial isolation from dropsy diseased carp shows that the disease is related to a mixed infection with Aeromonas and Pseudomonas bacteria.

According to Hasan et al. (2014), aquaculture development is currently following an increasing trend due to the increase in commercialization of aquatic product to fulfil the demand for food security following the growth of human population. The relevance of the aquaculture sector for development can be seen in the study by Little et al. (2016), which estimate that 120 million livelihoods are supported by fisheries-related activities, including jobs in supply networks (Little et al., 2016). This gives ways for the importance of aquaculture industry. According to Naylor et al. (2021), food security and global supply volumes are contributed mostly by the inland aquaculture, particularly in Asia as well as being a promising alternative to the global decline in fisheries stocks.

Despite having such importance, however, a growth in aquaculture activities correlates to an increase in the likelihood of serious disease problems in aquaculture fish, such as dropsy. Disease outbreaks have a severe influence on aquaculture fish yield, but the economic losses caused by these issues are difficult to quantify (Dias & Martins, 2017).

Several aquaculture fish species which includes common carp, silver carp, Nile tilapia, koi and catfish have been reported to be affected by the occurrence of dropsy.

Empurau or scientifically called as *Tor tambroides* is a highly profitable riverine fish in Southeast Asia that is relatively new to aquaculture sectors. The interest in aquaculture of this fish species stems from its critical cultural and socioeconomic relevance as food, ornamental, and sporting values (Lau et al., 2021). Because of the loss in natural population and distribution of *Tor tambroides*, there has been a surge of attention in artificial propagation, for both aquaculture production and preservation of this species (Asaduzzaman, 2016). According to Ingram et al. (2005), the commercial value of this species in the fish market can be as high as RM 400 per kg. In Sarawak, the price of this species has recently ranged from RM800 to RM1,000 per kg (Bernama, 2021). This means that the economic value of this species has improved exponentially throughout the year.

The present study aims to perform microbial profiling on healthy and dropsy diseased Empurau to provide the specific data on normal microflora of healthy Empurau and pathogenic agents of dropsy in Empurau, respectively. From the profile, it is expected that the diseased Empurau will contain the pathogens of the same strains and variety which causes dropsy in other fishes. Furthermore, the resulting profile can be used to assess how much dropsy influences the microflora of healthy Empurau through comparison with microflora of dropsy Empurau. These data can be used in the future study to prepare for precautionary steps and treatment to avoid the growth of the causative agents in aquaculture environment. This will increase Empurau production in aquaculture habitat and produce high quality fish yield of this species. Therefore, the objectives of this study are;

1) To characterize and profile the bacteria in the guts of dropsy diseased Empurau.

2) To identify the bacteria based on their 16S rDNA gene.

3) To compare the microflora on the guts of healthy Empurau with dropsy diseased Empurau.

CHAPTER 2

LITERATURE REVIEW

2.1 Empurau

Empurau or locally known as Kelah with its English name, Malaysian Mahseer (*Tor tambroides*) are the most valuable indigenous fish species to Sarawak, East Malaysia (De Silva et al., 2004). Early descriptions of *T. tambroides* species had few or no distinguishing traits with another Tor species of peninsular Malaysia known as *Tor tambra*, which explains why secondary literature on *T. tambroides* species taxonomy and systematics is limited (Walton et al., 2016). Recently, Jaafar et al. (2021), in his publication stated that, the IUCN Red List of Threatened Species (Version 2020-3) classified *Tor tambroides* as a valid species with unknown population status and their species status as data deficient.

According to Lau et al. (2021), the shape, size, and length of the median lobe are the distinguishing features of species within the genus *Tor*. This species can be distinguished by its silvery-bronze body colour and reddish dark fin with upper median projection, long lower median lobe, and pointed rostral hood as distinct physical traits (Jaafar et al., 2021). *T. tambroides* have great economic importance, particularly in Sarawak, due to their distinct flavour, high nutritional content, and diet based on a native plant of Borneo Island, Engkabang fruit (Lau et al., 2021). Because of their diet on Borneo's indigenous flora, this species has become more precious in terms of market value.



Figure 2.1 Tor tambroides 168.0 mm SL, Sarawak (Adapted from *The Non-Native Freshwater Fishes of Singapore: an annotated compilation*, by Tan et al., 2020).

The worth of this fish species in terms of market and share value has been recognised, and various studies have been conducted to improve *T. tambroides* production. According to Ingram et al. (2007), a breeding programme to develop artificial propagation and culture techniques for this species in 2007 was commence by Department of Agriculture Sarawak for conservation and aquaculture reasons. In fact, more research has been conducted since then. In recent years, research works on the genus Tor have expanded, with a heavy emphasis on aquaculture (Pinder et al., 2019) which involve the *T. tambroides* species.

In 2012, Department of Agriculture Sarawak mentioned that expansion of production capacity for *T. tambroides* through aquaculture development will be a valuable export trade for Sarawak. In fact, a report by Lam (2021) shows that *T. tambroides* have significantly high export value in countries such as Singapore, Hong Kong, China and Taiwan but are slightly low demanded in the local market due to their exorbitant costs. As a result, the growth of aquaculture in *T. tambroides* will allow for more favourable prices for this species' market value while also increasing their market size in both the global and local markets. More extensive research on the breeding and maintenance of this species' growth is indeed required in the near future.

2.2 Dropsy disease

IAD, like other diseases, can be caused by a variety of factors and is recognized by the symptoms. The common signs of infectious dropsy, according to (Aly & Ismail, 2016), are haemorrhagic lesions in the skin, eye, fin and tail where some fish showed signs of scale loss, slow movement, and imbalance. The infected fish is also claimed to have septicaemia symptoms such as congestion and haemorrhages in the internal organs and skin, as well as enlarged anus, ascites and exophthalmia (Aly & Ismail, 2016). To support this, an earlier study by Sharma et al. (2012), define dropsy as an infectious illness characterised by the scale protrusion, build-up of fluid within the body cavity, haemorrhagic sores on skin and fins, exophthalmic condition and intestinal inflammation.

Edun (2007), claimed that the most common symptoms of infectious abdominal dropsy noticed at the time is swelling of the body in the ventral region which gave rise to the name of this ailment. In 2011, a study on the pathology of dropsy by Haryo and Nurhidayati (2011), confirmed that this disease can be simply identified by looking at the swollen physical condition of the fish, together with the previously mentioned symptoms. Haryo and Nurhidayati (2011), also stated that the high mortality rate and strong potential for this disease to spread to other fish can cause economical losses for farmers. Nonetheless, no work has yet to focus on the economic losses caused by dropsy disease in *T. tambroides* species.



Figure 2.2 Tor tambroides with dropsy disease displayed (A) swollen abdomen with early sign of haemorrhaging (redness) under the skin (B) bulging eyes and abnormal appearance of scales

2.2.1 Cases of dropsy in aquacultured fish

Infectious dropsy has been a problem as aquaculture disease for decades, which raise the interest of researchers to further explore about this illness. Based on Densmore (2019), dropsy is common among pond farmed and hobbyist cyprinids, despite the fact that it is not endemic to any taxonomic group of fish. The occurrence of dropsy has been seen and studied since 1930 as mentioned by Kumar et al. (1986) where it can be found in European carp and Indian major carps where the first bacterial isolation was made in 1972. An experiment conducted by Haryo and Nurhidayati (2011), shows there are signs of dropsy infecting Koi fish in Indonesia fish farms, concluding that poor water conditions can lead to the disease's prevalence.

In 2015, Aly and Ismail (2016) discovered a large number of clinical dropsy cases in Common carp (*Cyprinus carpio L.*) from multiple private fish farms in part of Egypt known as Sharkiya province. The presence of the disease has had a significant impact on Egypt's economy and carp output (Aly & Ismail, 2016). Infectious dropsy has played a minor role in the average loss of fish productivity in India's selected aquaculture ponds, particularly of carp and catfish species (Mzula et al.,2019). Dropsy is also listed by Sharma et al. (2012) as a common occurring disease in fish. Densmore (2019) stated that dropsy of both infectious and non-infectious type can be caused by several factors and mostly favours the Cyprinids fish but are also found in other fishes. Because the *T. tambroides* species is still relatively new in the aquaculture business, research on dropsy for this species is currently scarce.

2.3 Potential causative agents

Infectious diseases are recognised to have causal agents and to be exacerbated by external influences. Back then, the majority of authors have agreed that infectious dropsy, specifically in carp is contagious, however the causative agents for the diseases is debatable

(Kumar et al., 1986). From the article, Kumar et al. (1986) mentioned that most workers and researchers thought it was a bacterial infection, some thought it was a viral infection, and yet others thought it was a product of virus and bacterial infections. In 2007, an investigation by Edun (2007) shows that *Aeromonas hydrophila* was recovered from a sample of diseased and dead fish that had infectious dropsy.

Bacterial isolation done by Aly and Ismail (2016) shows that the incidences of infectious dropsy in their study area was a mixed infections associated with *Aeromonas hydrophila* and *Pseudomonas fluorescens*. Densmore (2019) emphasized that dropsy as an infectious disease, which process are linked with bacterial or mycotic and viral infection that are associated by pathogenic pseudomonad, aeromonad and vibrio bacteria. Another of the most well-known causal agents responsible to cause dropsy in cyprinids and other fish is *Rhabdovirus carpio* (Densmore, 2019).

In accordance to a study by Faruk and Anka (2017), environmental factors such as organic pollution, temperature variations, overpopulation, poor water quality and inadequate nutrition of fish farms could readily promote the early stages of bacterial infections. This has been the key factor in the spread of *A. hydrophila*, a pathogen that causes dropsy, mostly of juvenile Indian major carps (Faruk & Anka, 2017). Sharpe (2020) claimed that the agent that induces the symptoms of dropsy is frequently the common *Aeromonas*, the gram-negative bacteria. Whilst various study on the causative agents of dropsy have been reported in different fish species, there remains a need to determine the actual causative agents of dropsy in *Tor tambroides* as it may yield a different result. Ultimately, aeromonads bacteria are expected to be the most possible microbes found in dropsy diseased *T. tambroides*.

2.4 Microbial profiling

Currently, the housekeeping genes are found to be very useful in molecular identification of bacterial species due to their high discriminatory and resolving power for precise identification (Mzula et al., 2019). This method has been extensively used as molecular assay in microbial profiling by sequencing the housekeeping gene, 16s rDNA to discover the microbes present in a sample (Qaadri, 2017). Being one of the most crucial methods in identification of microbes as mentioned by Zhou and Li (2015). A quantitative measure on the microbes can be provided through the use of microbial profiling assay (Qaadri, 2017), which will provide the right morphological and molecular data needed in this study.

There are several methods or procedures that can be utilized in order to complete profiling of microbes in diseased fish. In term of physical or morphological characterization, gram staining and microscopy are the common methods used. Gram staining, according to Tripathi and Sapra (2021), entails the use of methylene blue or commonly the crystal violet as the primary colour, having the gram-negative organisms not take up the primary strain where they appear reddish-pink in colour under a microscope. The cell walls of Grampositive bacteria have a peptidoglycan, thick covering of protein-sugar compounds and have poor lipid content (Aryal, 2021). Upon decolorization, the thick cell wall will dry and shrink which then close the pores in the cell wall and blocking the stain from exiting the cell (Aryal, 2021), leading to the formation of purple or blue gram-positive bacteria (Tripathi & Sapra, 2021).

In clinical microbiology laboratories, 16S rDNA sequencing has been critical in the correct identification of isolated strains of bacteria and the detection of new microorganisms. In the case of bacteria with uncommon morphological profiles, culture-negative illnesses, slow-growing bacteria, rare bacteria and non - cultivable bacteria, 16S rDNA

sequencing is especially helpful for their identification (Woo et al., 2008). According to Lopez et al. (2014), the procedure of 16s rDNA is very useful for genus classification and can substitute the more time-consuming transformation procedure, but it is of limited usefulness for identification of species. The sequence of this gene is typically too conserved to characterize a bacterial species, according to Stackebrandt (2014), but after the species has been defined, its analysis accelerates the identification process. In the past, obtaining information on superficial resemblances to known species required time-consuming methodology (Stackebrandt, 2014) which are now can be obtain easily through the use of 16s rDNA assays.



Figure 2.3 The summary on the example of procedure for each morphological and molecular methods used in microbial profiling assays.

2.5 Multi-antibiotics resistant bacteria

Referring to the description by World Health Organization (2022), bacteria that are no longer respond to medicine due to changes over time and make infections harder to treats, having the ability to increase the spread of disease are called as antimicrobial resistance. This means that, multi-antibiotics resistant bacteria can be identified when the bacteria can resist more than one antibiotic. This makes them to be more difficult to treat and have high tendency to transfer this resistance genes to other bacteria over some period of time, leading to global environmental threat (Watts et al., 2017). The emergence of multi-antibiotics resistant bacteria is found to be the result of large amounts usage of antibiotics in farm animals as well as in aquaculture industry (Nikaido, 2009). The issues regarding this multi-drug resistant bacteria rise as they can cause poor consequences in the natural environment and public health if they are able to spread and enter human food chain (Nguyen et al., 2021). The study by Nguyen et al. (2021) also proves that, treating bacterial infections in aquaculture using excessive amount of antibiotics has caused detrimental effects, making fish and aquatic animals to be accumulated with drugs and inhibit their growth.

According to Nikaido (2009), there are two possible mechanisms by which the multiantibiotics resistant bacteria may be developed. The first mechanism involves the accumulation of multiple genes in these bacteria, where each code for single drug resistance within a single cell (Nikaido, 2009). The accumulated multiple genes usually happen on resistance plasmids (Nikaido, 2009). This mechanism can be further classified as acquired resistance when some strains of bacteria with this resistance plasmid transfer their genes to other bacteria that is normally sensitive through the process of conjugation, transduction or transformation (Caruso, 2016).

Based on Nikaido (2009), the second mechanism are associated with genes, coding for efflux pumps of multidrug that is increasingly expressed causing a wide range of drugs to be forced out. This means, this mechanism applied in bacterial species that is readily resistance to an antibiotic due to the compounds' inability or low affinity to the target site (Caruso, 2016). This mechanism is called as intrinsic resistance mechanism. In accordance to Caruso (2016), generally, the emergence of antibiotic resistance genes is the product of modified genetics on natural defence mechanism of bacteria that is triggered to endure the drug activity and in other required condition. The study on the status of antibiotic resistance have been done on various isolates from different fish type in multiple fish farms. Based on the study by Nguyen et al. (2021), 13 bacterial strains isolated from *Anabas testudines* fish in Ho Chi Minh City, Vietnam shows several multi-antibiotics resistance bacteria classified according to CSLI standard. The result of the study shows that, the highest number of antibiotics can be resist is 7 out of 10, by one of the bacterial strains from the isolates and there are three bacterial strains that is resistance to 6 out of 10 antibiotics (Nguyen et al., 2021).

Another study by Fauzi et al. (2021) shows that there are several multi-drug resistant bacteria appeared from 61 isolates that consist of *Aeromonas* spp.cultivated from freshwater fish in Terengganu and Kelantan. A total of 44 out of 61 isolates were multi-resistant towards 14 antibiotics used in the study with all isolates are resistant to ampicillin (Fauzi et al., 2021). Furthermore, there are four isolates that are resistance up to 9 antibiotics. This further proved that multi-resistance bacteria have been increased over time. Nonetheless, there remains a need to statistically identify how much the emergence of multi-drug resistance have effects to the ability of certain drugs in treating dropsy in aquaculture sectors.