Prevalence of *Streptococcus agalactiae* in Hybrid Red Tilapia (*Oreochromis sp.*) From Inland Farm in Jeli, Kelantan

By

Amirah Binti Mohd Arshad

A report submitted in fulfillment of the requirements for the degree of Bachelor of Applied Science (Animal Husbandry Science) with Honour

Faculty of Agro Based Industry

UNIVERSITI MALAYSIA KELANTAN

2018

DECLARATION

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

_____

Student

Name:

Date:

I certify that the report of this final year project entitled "_____

_____"

by _____, matric number _____ has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Animal Husbandry Science) with Honours, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

Approved by:

_____

Supervisor

Name:

Date:

ACKNOWLEDEMENT

First of all I would like to thank Allah S.W.T for making all this possible for me. Many people have contributed their precious time in the completion of this thesis and it probably took a whole new chapter for me to show how grateful I am for their support. I am especially grateful to my supervisor Miss Hazreen Nita Binti Mohd Khalid for the continuous support and guidance that she gave me throughout the entire process of research and writing. Sincere thanks also extended to all the staff especially to the laboratory assistant of Faculty of Agro Based Industry, Mr. Wan Syamsul who helped me a lot in the laboratory during the experiment. I am also grateful to all the people who I met at National Fish Health Research Centre (NaFish). I am extremely thankful and indebted to them for sharing their place, time, expertise and valuable guidance extended towards me. Much love goes to my parents, sister and brother for providing me unlimited support and encouragement for me to successfully completing my experiment. Your love, affection and inspiration sustained me throughout all the years of study. Research sometimes is a lonely and frustrating pursuit, and my thanks also goes to all those who offered and who kept me company during my studies. A very big thank you to my beloved roommates and my happy pills, Fairus Elinna and Azammierah who never failed to cheer me up during my difficult times. Last but not least, thanks to all my friends and my course mate. To them all this work is dedicated.

Prevalence of *Streptococcus agalactiae* in Hybrid Red Tilapia (*Orechromis sp.*) From Inland Farm in Jeli, Kelantan.

ABSTRACT

Tilapia is a worldwide cultured fish both in freshwater and brackish water, and known as aquaculture chicken as it is present in all continents except Antarctic. Major problems in local tilapia breeding are mostly related to improper management system and lack of biosecurity that could introduce the occurrence of diseases. The outbreak of Streptococcosis had been reported more than a decade ago but up until today it is still known as common cause of mortality in tilapia population thus resulting in economic loss. The aim of this study was to screen *Streptococcus agalactiae* from hybrid red tilapia, *Oreochromis sp.* by molecular techniques. Subsequently, the prevalence of *S. agalactiae* infection in tilapia captured from inland farm located in Jeli, Kelantan was investigated. Four samplings was conducted where ten fish was randomly collected respectively. The DNA extraction was done and then subjected to polymerase chain reaction (PCR) for *S. agalactiae* specific 16S rDNA detection. Following the PCR optimization, one well defined band was chosen indicating the perfect annealing temperature for the reaction at 52.8˚C. All samples obtained from sampling showed no clinical sign of *S. agalactiae* except for enlarged kidney which observed in some of the fish during necropsy. Aside from showing the clinical sign of usual Streptococcosis infection, no positive PCR result was obtained indicating that the fish is free from *S.agalactiae* infection.

Keyword: Tilapia, *Streptococcus agalactiae*, polymerase chain reaction (PCR), molecular techniques.

Prevalens *Streptococcus agalactiae* Dalam Tilapia Merah Hibrid (*Orechromis sp.*) Dari Ladang Daratan Di Jeli, Kelantan.

ABSTRAK

Tilapia adalah ikan yang diternak dalam air tawar dan air payau diserata dunia, dan dikenali sebagai ayam akuakultur kerana ia terdapat di semua benua kecuali Antartika. Masalah utama dalam pembiakan tilapia tempatan adalah kebanyakannya berkaitan dengan sistem pengurusan yang tidak betul dan kekurangan biosekuriti yang dapat meyumbang kepada kejadian penyakit. Wabak Streptococcosis telah dilaporkan lebih dari satu dekad yang lalu tetapi sehingga hari ini masih dikenali sebagai penyebab kematian dalam populasi ikan tilapia sehingga mengakibatkan kerugian ekonomi. Tujuan kajian ini adalah untuk menyaring *Streptococcus agalactiae* daripada tilapia merah hibrid, *Oreochromis sp.* menggunakan teknik molekul. Seterusnya, prevalens jangkitan *S. agalactiae* pada tilapia yang ditangkap dari ladang daratan yang terletak di Jeli, Kelantan telah diselidik. Empat persampelan telah dijalankan di mana sepuluh ikan dikumpulkan secara rawak. Pengekstrakan DNA telah dilakukan dan kemudiannya menjalani tindak balas rantai polimerase (PCR) untuk pengesanan 16S rDNA khusus bagi *S. agalactiae* . Sejurus daripada pengoptimuman PCR, satu jalur yang paling baik telah dipilih menunjukkan suhu penyepuhlindapan sempurna untuk tindak balas pada 52.8˚C. Semua sampel yang diperoleh daripada persampelan menunjukkan tiada tanda klinikal *S. agalactiae* kecuali pembesaran buah pinggang yang diperhatikan dalam beberapa ikan semasa nekropsi. Selain menunjukkan tanda klinikal jangkitan Streptococcosis yang biasa, tiada hasil PCR positif diperolehi yang menunjukkan bahawa ikan tersebut bebas dari jangkitan *S.agalactiae*.

Kata kunci: Tilapia, *Streptococcus agalactiae,* tindak balas rantaian polimerase, teknik molekul.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
DEPC	Diethyl pyrocarbonate
MgCl	Magnesium chloride
dNTP	Deoxynucleotide triphosphate
TBE	Tris/Borate/EDTA
TB	Tris-Borate
UV	Ultra violet
rDNA	Ribosomal DNA
ATCC	American Type Culture Collection
SE	Standard error
α	Alpha
β	Beta
γ	Gamma
A	Adenine
T	Thymine
G	Guanine
C	Cytosine
°	Celcius
%	Percent
sp.	Species
G	Gram
m^3	Meter cubic
kcal	Kilo calories

kb	Kilo base
µl	Microlitre
ml	Mililitre
mg	Miligram
pb	Base pair
pH	Potential of hydrogen

CHAPTER 1

INTRODUCTION

1.1 Research Background

The aquaculture industry has grown rapidly in recent years. Started from 1990s, aquaculture industry in Malaysia has been developed and is now become one of the important economic activities in industry (Kechik, 1995). Numerous types of culture practices are used with variety of species are cultured such as marine fish, freshwater species and also shellfish. Nowadays aquaculture has become nation important sector as total revenue increases significantly with the increase in local demand for food security. Unfortunately, difficulties in acquiring a suitable land, rise in daily production costs, unskilled labour and diseases threat are the problems which obstruct the aquaculture development and growth (FAO, 2010).

Tilapia is one of the commercially important fish in Malaysia as well as in other parts of the world. Generally, tilapia is classified into three different genera, *Oreochromis, Sarotherodon* and *Tilapia* based on their distinguishing characteristic of taking care their fry (Abdelhadi, 2011). Parents of the genera Sarotherodon and Oreochromis, will incubate and protect their young in mouths which namely mouth brooding. In Sarotherodon, either male or female are mouth brooders while in Orechromis, only female practice mouth brooding. Important commercial species are

include the Mozambique or Java tilapia (*Oreochromis mossambicus*), blue tilapia (*O. aureus*), Nile tilapia (*O. niloticus*), Zanzibar or Wami tilapia (*O. hornorum*), and the redbelly tilapia (*O. zilli*). The hybrid red tilapia (*Oreochromis sp*.) is the hybrid result of crossing between Java tilapia (*O. mossambicus*) and Nile tilapia (*O. niloticus*) (Chapman, 2015).

Streptococcosis is a lethal bacterial disease in fish that causes economic losses in freshwater and marine fish production, which is economically important in many countries, including Malaysia. Streptococcosis can be spread either due to the recurrence of disease at the same place or emergent to new place as a result of poor husbandry management and improper biosecurity in the farm (Thai Agricultural Standard, 2009). Streptococcus species that could cause disease in fish include *Streptococcus agalactiae*, *S. dysgalactiae*, and *S. equi, S. equisimilis, S. faecium, S. pyogenes, S. zooepidemicus* and *S. iniae*. From all the species mention, *S.iniae, S.agalactiae* and *S. dysgalactiae* are the major disease causing pathogen of aquaculture species that could resulted in severe economic loss in aquaculture industry.

Inappropriate management practices or abrupt change of the environment is the main predisposing cause of Streptococcosis infection in fish mainly tilapia. It is reported that a relatively high temperature more than 30° C can induce stress in fish and favorable for bacterial growth (Ndong, Chen, Lin, Vaseeharan & Chen., 2007). Bacteria may enter the fish by cannibalism between fish where fish consume moribund or dead fish which were several organs (Watanabe, Losordo, Fitzsimmons, & Hanley, 2002). Infected tilapia had shown a sudden erratic pattern of swimming and unbalancing body besides exophthalmia, or haemorrhages in skin surrounding

the anus or at the base of the anus (Zamri-Saad, Amal, Siti-Zahrah, & Zulkafli, 2014). Internally, the brain become watery soft and a pale enlarged liver were observed (Musa et al., 2009).

1.2 Problem Statement

The major problem in local tilapia breeding is mostly related to improper management system and lack of biosecurity. These two practices are important to prevent and reduce the introduction and the occurrence of the disease to another place. The outbreak of Streptococcosis have been happen more than a decade ago but up until today it is still known as common cause of death in tilapia population thus resulting in economic loss. The continuation or the recurrence of the disease also means that the *Orechromis sp.* could possibly be harm on food safety and causing a foodborne illness or poisoning in human which who consume the infected fish. Thus, early detection of the disease is suggested so that specific measure can be taken and economic loss can be minimized.

1.3 Objectives

The purpose of this study includes:

1. To screen the presence of *Streptococcus agalactiae* in Hybrid Red Tilapia (*Oreochromis sp.*) using molecular techniques.
2. To determine the prevalence of the *S. agalactiae* infection from inland farm in Jeli, Kelantan.

1.4 Scope of Study

The study focus on molecular biology in diagnosis of aquatic disease.

1.5 Significant of Study

Streptococcosis is a disease that develops following the infection by *Streptococcus sp.* It is one of the most dangerous, highly pathogenic bacteria in freshwater, marine and brackish water. In fish, *Streptococcus sp*. has been reported to cause considerable morbidity and mortality worldwide. Many studies have been carried out to reveal the transmissions of *Streptococcus sp.* and it is found that the main cause of the disease outbreak is the introduction of new fish in farm ecosystem. Besides, the bacteria excreted along the feces also can be the reason of the outbreak by exposing the bacteria to other healthy fish in the same pond.

This aim of this study was to screen the presence of *S. agalactiae* as the emergent fish pathogen that possibly cause the disease outbreak in tilapia. Therefore, finding of this study may provide the information that could be documented on streptococcus disease and its prevalence in inland farms located in Kelantan area that can be used for future reference. As the early detection of the disease is possible, prevention would be much easier as it provide benefits for the whole fisheries industry. Farm productivity and fish resources also could be increase tremendously.

CHAPTER 2

LITERATURE REVIEW

2.1 Hybrid Red Tilapia (*Oreochromis sp.*)

Freshwater aquaculture can be carried out in different types of culture systems accordingly such as ponds, abandoned mining pools, pen, cages or even tank. The use of ex-mining pools for freshwater fish farming is unique to Malaysia due to the many abandoned such sites that are now filled with water (Kechik, 2015). Fish are either released into these pools of various sizes and shapes or are farmed in small cages suspended in the water. Three major freshwater species farmed fish are tilapia, catfish and carp (Wing-Keong, Sih-Win, Kabir, Chowdhury, & Bureau, 2013). Unlike other countries where Nile tilapia is the major farmed species, Malaysia is known for its hybrid red tilapia, *Oreochromis sp*. which accounts value of 82 % of total tilapia production (Wing-Keong et al., 2013). *O. niloticus* and *O. mossambicus* was the original hybrid red tilapia introduced to Malaysia whereas hybrid red tilapia that has no known genetic status is bred for marketable purpose. A total of 104 farms were surveyed throughout Malaysia including farms located in Sabah, Perak, Selangor, Negeri Sembilan and Sarawak than in others (Wing-Keong et al., 2013).

Nile tilapia (*O. niloticus*) that were introduced by Indonesia in 1944, showed the highest total number of aquaculture production, followed by catfish and carps (Ang, Gopinath, & Chua, 1989). The black nile tilapia, introduced back in 1950's, did not perform well in terms of growth and also production due to its unattractive presence compared to hybrid red tilapia introduced by land around 1979 (Ang, Gopinath, & Chua, 1989). The 2001 collaboration formed with World Fish Centre has achieved a significant outcome in successfully produce all male tilapia by genetically improved fish tilapia (GIFT), indicating the beginning of culture of all males or monosex tilapia. In terms of production, male tilapia grows faster and capable of achieving a uniform in size compared to females which is slow in growth. For this reason, monosex tilapia farming can be done by manually sexed, direct hormonal sex reversal, manipulation of genetic or hybridization which has been testified as a solution to early sexual maturation problem and undesirable reproduction (Gupta & Acosta, 2004).

The hybrid red tilapia, was first produced by interspecific cross between albino *O. mossambicus* and *O. niloticus* in Taiwan resulting in production of '3rd generation of tilapia' that have better appearance of colour and other desired features (Anon, 1984). In terms of performance, Alceste, (2000) claimed that hybrid red tilapias are suitable for brackish water and seawater culture because of salinity tolerance of the parental species, known to be moderately *O. niloticus* and *O. aureus* to highly euryhaline *O. mossambicus* and *O. hornorum*. Growth rates of tilapia are influenced by multiple depending factors such as water temperature, sex, supplemental feeding, and stocking. Tilapia is prone to cold water temperatures and would stop eating when the water temperatures began to drop below 16˚C, followed by dying at around 13°C. Preferred water temperature for intensive culture of hybrid red tilapia is between 25˚C to 32˚C (Chapman, 2015).

Stocking density recommended for table size tilapia varies accordingly in fish size and production system. In fed and aerated ponds, young hybrid tilapia of 50g will be stocked at 9,500-19,500 fish per hectare. For tanks or raceways system, 25g–50g tilapias are stocked at 140 and 248 fish/m^3 densities in container space. As in grow-out production cages, 60g–100g of hybrid tilapia range can be stocked from 250-400 fish per cubic meter (7–11 fish/ft^3) of cage. If the cages are to be placed in ponds, the stocking density of the pond must not exceed those numbers which would be achieved by growing the fish free-swimming in the pond (Chapman, 2015).

Commonly, hybrid tilapia usually marketed as red or golden tilapia where fresh tilapia are sold in the range of 450g to 680g and approximately content 30% to 39% whole fish to boneless fillets. Fish often being marketed as whole, fresh or even frozen. Nutritive value of hybrid tilapia is considered around 96 kcal/100 grams of raw meat, 19.2% protein and 2.3% fat by weight (Chapman, 2015).

2.2 Streptococcosis

Streptococcus is a septicemic disease caused by different Streptococcus species. It has caused a great loss economically of the marine and freshwater fish production in many countries including Malaysia. Most of the Streptococcus is oxidase-negative and catalase-negative. While many streptococci are facultative anaerobes, there are some of them are obligate anaerobes. Most of these bacteria require enriched media as a medium for its growth such as blood agar.

Streptococci can be categorized based on their colony morphology, biochemical reactions, type of hemolysis and also by serologic specificity. They are divided by three groups differentiated by hemolysis on blood agar: beta (β), alpha (α) and gamma (γ) hemolytic. Oxidation of iron in hemoglobin compound occurs as a result of alpha hemolytic thus displaying green colour on the surface of blood agar. Beta-hemolytic species showed a clear area of blood cells surrounding the colonies of bacteria in blood agar resulted from whole breach of the red blood cells. No hemolysis occurred by Gamma-hemolytic species (Public Health England, 2014).

Streptococcus agalactiae was first recognized in 1939 in the mid-twentieth century where it is known an important disease causing pathogen in human. Few decades before, *S. agalactiae* has often been isolated from a variety of non-human sources and is now well recognized as an important contributor of zoonosis disease (Sun et al., 2016). Hence, this makes the authorities realizes that they might be overlook this matter as streptococcus infection pose a big threat to the fish farming industry, especially the tilapia industry. This might be due to the increasing demand and the production from the industry as well as the trading of tilapia all over the world.

Streptococcosis outbreaks in tilapia were reported in many countries, such as the United States of America, South Africa, Japan, Israel, Italy and Thailand (Thai Agricultural Standard, 2009). The disease was found in black and red tilapia, of all sizes. The disease occurrences were reported in all regions of tilapia producing countries. The first outbreak in Malaysia was reported in 1997 at the Pahang river where *S. agalactiae* infected hybrid red tilapia (Zamri-Saad et al., 2014). The first outbreak was seen to be happened on tilapia on average weight of 300g-400g which

ended with 60% mortality. Subsequent outbreak in March and June 2000 occurred where *S. agalactiae* were reported to infect a population of tilapia at Lake Kenyir, Terengganu and Lake Pergau, Kelantan where almost half of the population died (Zamri-Saad et al., 2014). Today, streptococcosis have been reported at almost all area in peninsular Malaysia.

Disease pattern and severity are different depending on bacterial strains and farm management system. Abrupt change in farm management, intensive farming, improper harvest or handling of fish, resulting in skin laceration, as well as poor water quality, such as low dissolved oxygen, high ammonia or nitrite, induce stress which has negative impact on fish immunity.

2.2.1 Disease transmission

The main cause of streptococcosis is mainly because of the improper management practice and also abrupt change in temperature that favour bacterial growth (Amal et al, 2013). Factors and stressors that increased the susceptibility of fish to streptococcal infection includes introducing polluted water or new unknown fry into the farm, high stock density, poor farm biosecurity, declining of water qualities such as slow flowing water, high water temperature, high ammonia content, low dissolved oxygen, unsuitable pH and salinity in the culture system (Pongthana et al, 2010). Fish react to the infection by instigating inflammatory process, which counterbalance pathogens by white blood cells such as macrophage and polymorphonuclear leukocyte. Cell-mediated and humoral immunity also play a vital

role in preventing the bacteria to reach blood circulation and internal organs (Thai Agricultural Standard, 2009).

However, some of the bacteria are able to evade the immune system using its particular properties to cause systemic infection for instance, Surface antigens of Streptococcal bacteria can attach to the fish's cell surface, which prevent them to be eliminated by fish's lysozyme. Consequently, the bacteria replicate in lymph and blood (septicemia) and spread to target organs, such as liver, kidney, spleen and brain (Baeck et al, 2006). Toxin production, the important hemolytic toxin of Streptococcus is streptolysin, which is further classified as streptolysin S and streptolysin O. The toxins cause complete hemolysis on blood agar. Streptolysin S produces hemolysis on the surface of blood agar (surface hemolysis), whereas streptolysin O produces hemolysis under anaerobic condition, in a deeper layer of blood agar (deep hemolysis) (Thai Agricultural Standard, 2009). The toxin rapidly damages cells and tissues, including white blood cells, liver and heart. Next is enzyme production, most of the enzymes produced by Streptococcus are capable of digesting large molecules, such as fibrin clump and connective tissue. This enables the bacteria to easily penetrate the tissue, especially at skin lacerations and mucous membrane of several organs (Patterson 1996).

All of the previous studies already discussed about the route of transmission of *Streptococcus sp.* Nguyen *et.al* (2002) state that introduction of new fish to the farm as one of the factor that cause the introduction of *S.iniae* and *S.agalactiae* into the farm. Besides, feeding of infected trashed fish could also contribute to the possibility of an outbreak to which already happened on flounder in Korea (Kim *et al*., 2007). In addition, Streptococcus transmission also is likely to occur within the same

aquatic environment where two different species of fish is cultured (Evans et al., 2002).

2.2.2 Clinical sign

Clinical signs of Streptococcus infected fish commonly detected in larger fish weight more than 300 g. There were two forms of clinical sign shown which are acute and chronic. Acute symptoms including non-directional, imbalance and spiral swimming. While gross signs include erratic swimming behavior with eye abnormalities either bilateral or unilateral exopthalmia, corneal opacity or cloudiness and hemorrhagic eyes in one or both eyes (Evans et al., 2002; Siti-Zahrah, Padilah, Azila, Rimatulhana, & Shahidan, 2008). Moreover, hemorrhagic lesions at the base of the fins, operculum, skin and tissue around ventcan also can be found. Significant internal lesions of the disease are presence of clear, viscous to hemorrhagic fluid in abdominal cavity, pale and enlarge liver, darken and enlarge spleen, hemorrhage and congestion at mucosal side of the intestine (Thai Agricultural Standard, 2009). These gross lesions are clearly visible in relatively large fish, however, they may be absent from infected juvenile or larvae. Mortality rate of the moribund fish may reach up to 50% within 3-7 days post infection or since when the first clinical sign was observed. Mortality rate may reach up to 80%-100%, in case of the severe outbreaks (Thai Agricultural Standard, 2009).

Chronically, tilapia may be found floating. Black colour tilapia shows a darkening body colour while red tilapia shows pale body colour. External lesions may include presence of pustules at caudal peduncle or under the month. Internal lesions

may include fibrinous peritonitis, adhesion of internal organs, pericarditis and/or absence of internal lesion. Mortality rate of chronically infected fish is low or none while feed intake are reduced, which prolong the culture period. Prolong culture period means loss in production economically and the carcass produced usually abnormal, defected thus, result in low meat quality (Behrends et al, 1982).

2.2.3 Preventive measure

Streptococcosis infection sometimes cannot be evaded as it can present in the area surrounding of the aquatic environment. A farm could reduce the risks of disease outbreak by acquiring known origin of incoming stock and the stock should be quarantine upon arrival to the farm. Overcrowding can be reduce by understanding stocking density and animal welfare, unnecessary feeding must be avoided, use separate supplies of water for culture system, restricted handling only to authorize worker or unnecessary transportation, frequently remove dead fish bodies, provide pathogen free feed ration, and maintain good sanitary environment or good biosecurity measure in a farm could also be done (Inglis et al., 1993; Klesius et al., 2008). Besides, regular routine of sanitizing farm machine and equipment can help in reducing the risk of disease transmission.

2.3 Polymerase Chain Reaction (PCR) principles as diagnostic tool

The introduction of polymerase chain reaction (PCR) has been drastically changed biological science from the period it was discovered years back (Mullis, 1990). The procedure was widely used by scientists to identify diseases, replicate and sequencing the gene, carry out sophisticated quantitative and genomic studies in a very quick delicate routine. In PCR, a sequence is amplified from the first few copies where it occurs in a test tube. A variety source of DNA can be used for PCR test. In order for PCR to be able to generate enough copies to be tested by a formal laboratory method, only small part of DNA is needed thus, PCR is recognized as very delicate test (Garibyan & Avashia, 2013).

Each procedure of PCR test requires the presence of DNA template, primers, nucleotides, and DNA polymerase (Garibyan & Avashia, 2013). In favor for PCR product to be produced, DNA polymerase acts as a responsible enzyme that connects the individual nucleotides. The nucleotides comprise of four bases namely adenine (A), thymine (T), cytosine (C), and guanine (G) that are found in DNA (Garibyan & Avashia, 2013). A, T, G, C act as complementary bases to produce the product of PCR test. In each reaction, primer is responsible to specify the exact DNA product to be amplified. Primers are short strands of DNA with a definite complement sequence to the DNA targeted that is to be identified and amplified (Garibyan & Avashia, 2013).

Section of DNA is amplified by using PCR. On opposite strands of DNA, short oligonucleotide primers will hybridize to each end of the target region of DNA to be amplified. In each round of thermocycling reaction, the template DNA is denatured, primers will anneal to their complementary regions and polymerase enzyme may catalyses the addition of nucleotides to the end of each primer, thus producing new copies of the target region in each round (Cunningham, 2002).

At the present day, restriction enzyme digestion, probe hybridization and polymerase chain reaction (PCR) are those most used method in diagnosing fish diseases (Cunningham, 2002). The absence or presence of product by PCR test may be adequate to show infection status of the samples by any disease carrier pathogen. The used of PCR practically in diagnosing test does need significant consideration to development of suitable methodologies and authentication to ensure greatest possible sensitivity and specificity are obtained. Although there are some possible difficulties regarding this method, PCR often play an important part in molecular diagnosis of fish disease.

2.3.1 Analysis of PCR product

Polymerase Chain Reaction product can be visualized in two main methods, which are chemically staining the amplified product by dye such as ethidium bromide or by labelling the primer using fluorescent dye prior to amplification. Agarose gel electrophoresis is the most frequently used method for analyzing the PCR product. It is the easiest method which split up DNA products on the basis of size and charge. A

preset DNA product with known sizes are run at the same time on the gel as standardized molecular markers to help indicate the final size of the product.

2.3.2 Advantages and disadvantages of PCR

Polymerase Chain Reaction (PCR) is a simple technique that can be easily used and most importantly gives rapid result (Vogel et al., 2012). It is an extremely delicate test that has the ability to generate millions to billions of copy of a precise product for sequencing, cloning, and other analysis. However it has some limitations. As the procedure is very sensitive thus, any possibility of a contamination can probably give different interpretation of the products (Smith and Osborn, 2009; Vogel et al., 2012). When designing a primer for PCR, some prior sequence data are needed. Therefore, PCR can be used only to detect the presence or absence of a known pathogen or gene (Garibyan & Avashia, 2013). Another limitation is that the primers used for PCR could mistakenly carry out nonspecific annealing to almost similar, but not completely identical sequence, to targeted DNA.

2.4 Fish disease diagnostic and detection

Even under greatest environmental conditions, a well taken care fish without a sign of infection or injury can pose a danger by carrying pathogens that can increase risks in contagious diseases to spread in the fish populations (Altinok & Kurt, 2004). Clinical sign can only be seen when a stressful condition occurs. Under some conditions, the risk of stress could increases and significant proportion of stock

may be infected. Thus, detection of pathogen presence in infected fish is effective measure for diseases control that could be done. The probability of the successfulness in detection of pathogen is increased when test is conducted on large number of fish. Traditionally the disease diagnosis is carried out by cultivation of agar and then phenotypic and serological properties of the pathogen or histological examination (Bernardet et al., 1990; Pazos et al., 1996).

Developments and great advanced in molecular biology field has taken place and directed to a rapid growth in new way of diagnosing fish diseases. Molecular techniques can be used as a solution and increase sensitivity and specificity of pathogen detection. These techniques include the use of molecular tools such as polymerase chain reaction (PCR), restriction enzyme digestion, probe hybridization, in situ hybridization, and microarray (Altinok & Kurt, 2004). Since diagnostic techniques by molecular biology are faster and highly accurate compared to the other detection technique, early detection of pathogen can be done, thus diseases outbreak can be prevented. The more rapidly the identification of the pathogen can be conduct, the more it can benefit the economically. As a result, the population of the infected organisms can be determined and proper management program can be applied.

Molecular tools are gradually important to detection of fish disease. Complete genome of pathogen sequencing could allow great advances in study of biology, and improving diagnosis as well as control the pathogen outbreak (Altinok & Kurt, 2004). Progress in detection techniques helps in epidemiological studies as well as identification of presence of pathogen in fish during the outbreak. Hence, molecular

biology can be beneficial tool in diagnosis, control and the epidemiology of infectious fish diseases.

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials

3.1.1 Chemical and reagent

In this study, the chemical and reagent are used for DNA extraction and PCR. For DNA extraction, GF-1 Tissue DNA Extraction Kit was used. As for PCR, the reagent used was (DEPC water, 10x Taq Buffer, MgCl, dNTP mix, forward primer, reverse primer, and Taq DNA polymerase), ethanol, 6X loading dye, agarose powder, GelRed Nucleic acid gel strain, 1X TBE running buffer, and 1kb DNA ladder.

3.1.2 Equipment and apparatus

The equipment and apparatus that are used in this study are centrifuge, vortex, thermal cycler, thermomixer, autoclave and UV transilluminator, NanoDrop Spectrophotometer, electrophoresis set, dissecting set, 100 ml beaker, 200 ml beaker, pail, 10µl, 200µl and 1000µl micropipette, 2-20µl pipet tips, 20-200µl pipet

tips, 100-1000µl pipet tips, 0.2 ml PCR microtubes with attached cap, 1.5 ml tubes, ice box, and chopping board.

3.2 Methods

3.2.1 Fish sampling

A total of 40 hybrid red tilapia, *Oreochromis* sp., appear to be healthy fish were sampled from Agro Techno Park of Universiti Malaysia Kelantan (UMK), Jeli campus. Adult tilapia fish with initial mean weight 145.42 ± 2.03 g was selected for the study. The fish sampled was collected twice a month for four times and were screened for bacteria (particularly for *S. agalactiae*). The screening of these bacteria was done using extracted tissue from the fish kidney.

3.2.2 DNA Extraction

Tissue sample from kidney of the hybrid red tilapia was cut into smaller pieces of 20 mg each and extracted using GF-1 Tissue DNA Extraction Kit by vivatis Technologies. After that, 250 µl of Buffer TL was added along with 20 µl of Proteinase K to the sample followed by mixing by pulse-vortexing. Next 12 µl of Lysis enhancer was added followed by immediate mixing. The mixture was then incubated in water bath at 65˚C for 1 hour.

After the incubation period, 560 µl Buffer TB was added followed by mixing using vortexing until homogeneous solution was acquired. The solution was incubated at 65˚C for another 10 minutes. Next, 200 µl of absolute ethanol was added into the mixture and immediately mix. Approximately 650 µl of sample was transferred into the column and centrifuge at 5000 x g for 1 minute and the flow through were discarded afterwards. The column was then washed with 650 µl Wash buffer and centrifuged at 5000 x g for 1 minute and the flow through was discarded. Washing step was repeated once again. The column was centrifuged at 10000 x g for 1 minute to remove all the traces of ethanol completely.

The column was placed in a clean microcentrifuge tube. About 200 µl of preheated Elution buffer was added directly onto the column membrane and left at room temperature for 2 minute. The tube was then centrifuged at 5000 x g for 1 minute to elute the DNA and stored at 4˚C or -20˚C (vivantis Tecnologies).

3.2.3 Polymerase Chain Reaction (PCR)

3.2.3.1 Optimization of PCR

Optimization was the first process carried out before the actual PCR conducted. In this process, the suitable annealing temperature was predetermined in order to produce certain desired product of 1457 bp. This variable in this study was temperature. So, in order to determine the perfect annealing temperature for the reaction, the following protocol was used; was initial denaturation at 94˚C for 4

minutes, 34 cycles of denaturation at 94˚C for 30 seconds, annealing temperature ranged from 50˚C to 60˚C for 30 seconds, extension at 72˚C for 1 minute and final extension at 72˚C for 7 minutes.

3.2.3.2 Amplification of target DNA

The extracted DNA was be subjected to PCR for *S. agalactiae* specific 16S rDNA with forward primers 5′ GCT ATA CAT GCA GTA GAA CGC TGA 3′ and reverse primer 5′ ACG ACT TCA CCC CAA TCA TCT AT 3′. Each 25 µL reaction of PCR master mix solution was prepared which consist of 21 µL of DEPC water, 1 µL of 10X PCR buffer, 1 µL MgCl, 0.25 µL of mmol dNTPs, 0.25 µL of 10 µmol Forward primer, 0.25 µL of 10 µmol Reverse primer, and 0.25 µL Taq DNA polymerase.1 µL DNA template was added into the master mix solution. For each of the diagnosis, the test consist of positive control (*S.agalactiae* ATCC), negative control (DEPC water) and 1kb of molecular DNA ladder. The amplification was performed in thermocycler machine (Eppendorf, Hamburg, Germany), which the cycling conditions was as follows; initial denaturation at 94˚C at 4 minutes, 35 cycles of denaturation at 94˚C at 30 seconds, annealing temperature of 52.8˚C for 30 seconds, extension at 72˚C for 1 minute followed by the final extension at 72˚C for 7 minutes.

3.2.3.3 Gel electrophoresis

Gel electrophoresis was performed to identify DNA band on agarose. Agarose was prepared beforehand by mixing 0.6 g of 1.5 % agarose with 40 ml of 1X TBE buffer. The mixture was then microwaved for 1 minute until the clear mixture appeared. After that, the gel was stained with GelRed Nucleic acid gel strain and poured into the gel cast. 1 µL of 6X loading dye was mixed with 4 µL of PCR product except for positive control with 3 µL PCR product and then loaded into each well of harden agarose gel prepared earlier. Electrophoresis was run at 70V for 50 minutes in 1X TBE buffer. The gel was then viewed by using UV transilluminator and observed for expected band.

CHAPTER 4

RESULTS

4.1 Clinical Observation

A total of 40 fresh hybrid red tilapia with average weight 145.42 ± 2.03 g was collected during the sampling. Ten tilapia fish was sampled and dissected each time. Observations were made on external gross lesion of the fish during the dissection by looking for any abnormality at the overall anatomy of the fish.

Figure 4.1: Hybrid Red Tilapia.

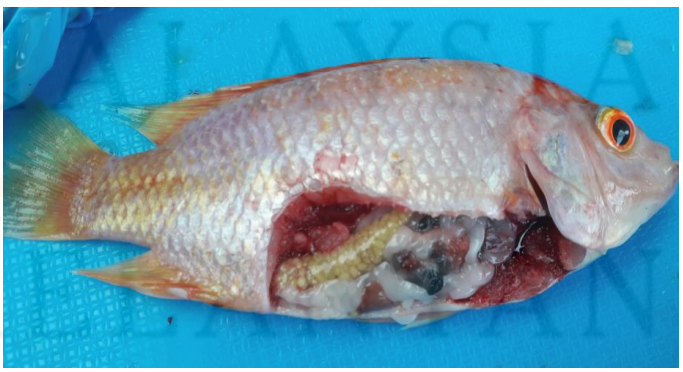

Figure 4.2: Internal anatomy of Hybrid Red Tilapia.

(a) (b)

Figure 4.3: Kidney condition. (a): Normal kidney of Tilapia. (b): Enlarged kidney.

During necropsy, the clinical sign was observed on the fish externally and some demonstrated enlarged kidney (Figure 4.3: (b))

Table 4.1: Average weight, standard error and internal organ observation of samples.

Sampling ID	Average weight ± Standard error (g)	Internal organ observation
1TAK	149.95 ± 4.22	2/10 enlarged kidney
2TAK	148.13 ± 4.28	2/10 enlarged kidney
3TAK	141.93 ± 3.85	1/10 enlarged kidney
4TAK	141.66 ± 3.81	1/10 enlarged kidney

Out of 40 samples of tilapia, six fish were observed having an enlarged kidney (Table 4.1). Other than that, most of the fish showed normal internal and external observation.

4.2 DNA Extraction

The screening process was started with DNA extraction using kidney tissue sample to extract out the DNA. For this procedure, DNA was extracted using GF-1 Tissue DNA Extraction kit and the quality as well as purity of the extracted DNA was measured by NanoDrop Spectrophotometer.

4.2.1 Quality of DNA

Nanodrop Spectrophotometer showed two readings of 260/280 ratio and 260/230 ratio. The first reading, 260/280 ratio indicates the absorbance of 260 nm and 280 nm used to purify DNA and RNA respectively. For DNA, a ratio of ~1.8 is acknowledged as pure DNA whereas for RNA, a ratio of ~2.0 usually indicates as pure RNA. As for the data recorded, extracted DNA showed quite good purity DNA data as the reading range was in ratio ~1.8 except for samples of Sampling 1(1TAK) that recorded with average of 1.66. If the ratio is significantly lower, it may indicate the presence of phenol, protein or any other contaminant absorb at or near 280 nm. (William, Karol & Piotr 1997).

Nucleic acid purity was measure by the second reading, 260/230 ratio. The reading of 260/230 for pure nucleic acid is slightly higher compared to 260/280 reading value. Common value for 260/230 reading is in the range of ~2.0-2.2. If the ratio is lower than the usual, it indicated the presence of contaminant which absorb at 230 nm.

Based on the result obtained from the reading of NanoDrop spectrophotometer, for 260/280 ratio, the purity of the DNA samples from 1TAK to 4TAK recorded between 1.02 to 2.17 with average of 1.87. While for 260/230 ratio, the purity of the DNA samples from 1TAK to 4TAK was recorded between 1.52-13.40 with average of 3.01. As a result, it can be concluded that DNA extracted was low in quality.

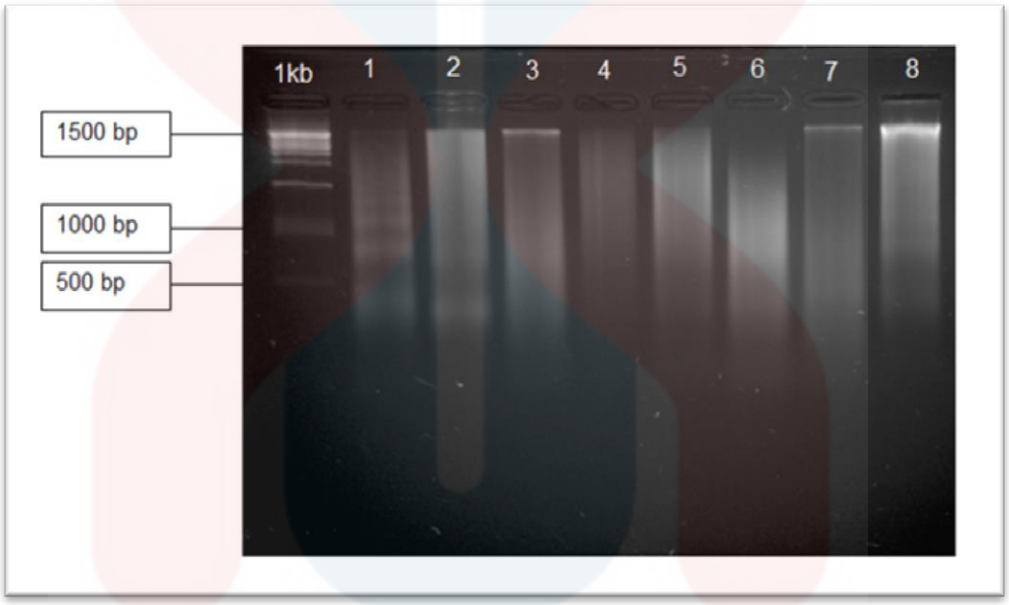

Figure 4.4: Result of gel electrophoresis on DNA sample. Molecular marker, 1kb DNA ladder; lane 1-2: DNA sample of 1TAK; lane 3-4: DNA sample of 2TAK; lane 5-6: DNA sample of 3TAK; lane 7-8: DNA sample of 4TAK.

4.3 Detection of *Streptococcus agalactiae* by Polymerase Chain Reaction (PCR)

In order to amplify specific region of DNA, PCR test was used (McPherson et al., 1991). At the opposite strands of DNA, specific primer was design in order to hybridize targeted region of DNA to be amplified. During each round of the thermocycling reaction, DNA template was denatured, primers anneal to their complementary regions and addition of nucleotides to end of each primer would be catalyzed by polymerase enzyme thus, new copies of targeted region was generated each time (Cunningham, 2002).

4.3.1 Optimization of PCR

Optimization is a process that is conducted at the very beginning of PCR procedure to adjust the variables in the reaction in order to produce single desired product. As one variable of PCR is changed, it may affect another and in this study annealing temperature is the variable that needs to be look into during optimization. The amplicon product expected to be produced was 1475 bp.

In this study, optimization mainly focused on temperature. The protocol used for this optimization was initial denaturation at 94˚C for 4 minutes, 34 cycles of denaturation at 94˚C for 30 seconds, annealing temperature ranged between 50˚C and 60˚C for 30 seconds, extension at 72˚C for 1 minute and final extension at 72˚C for 7 minutes.

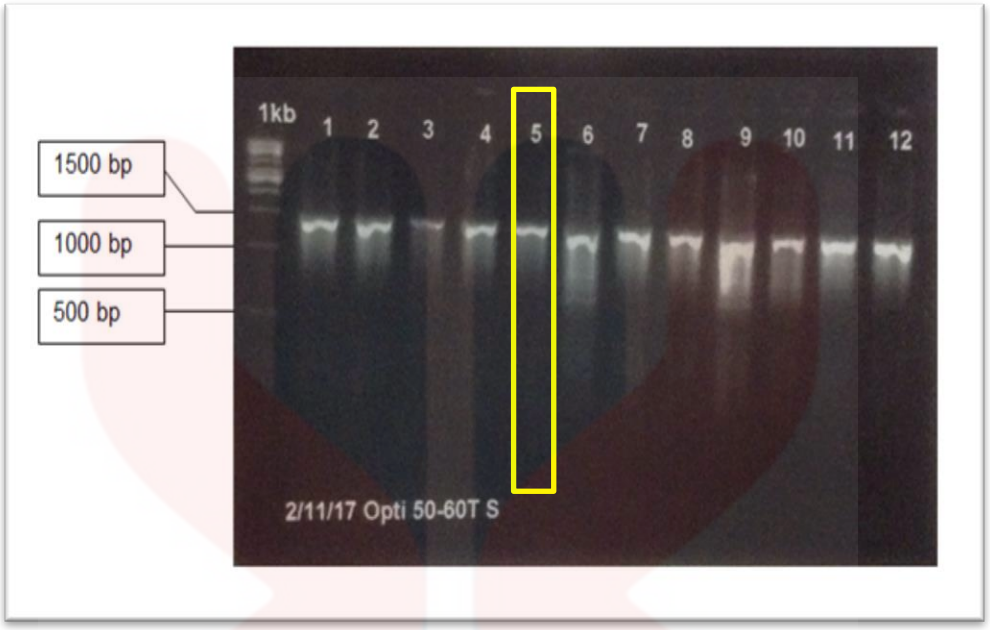

Figure 4.5: PCR optimization of varying annealing temperature. Sample used was ATCC 13813, 1kb DNA ladder as molecular marker; lane 1: 50.0˚C; lane 2: 50.2˚C; lane 3: 50.8˚C; lane 4: 51.7˚C; lane 5: 52.8˚C; lane 6: 54.1˚C; lane 7: 55.4˚C; lane 8: 56.7˚C; lane 9: 57.9˚C; lane 10: 58.8˚C; lane 11: 59.5˚C; lane 12: 59.9˚C.

From the result of gel electrophoresis, the best band which located at the 5th lane was chosen. So, the suitable annealing temperature was 52.8˚C as the lane produce brighter and more defined band compared to the other band that was brighter and thicker. The optimized protocol was subjected for PCR screening of *S.agalactiae* on all fish samples.

4.3.2 Detection of presence of *S. agalactiae* by using optimized PCR protocol

Result of PCR on 1TAK, 2TAK, 3TAK and 4TAK samples showed no detection of *S.agalactiae.* No expected band observed from lane one to lane ten aside from the band observed in lane PC with positive control.

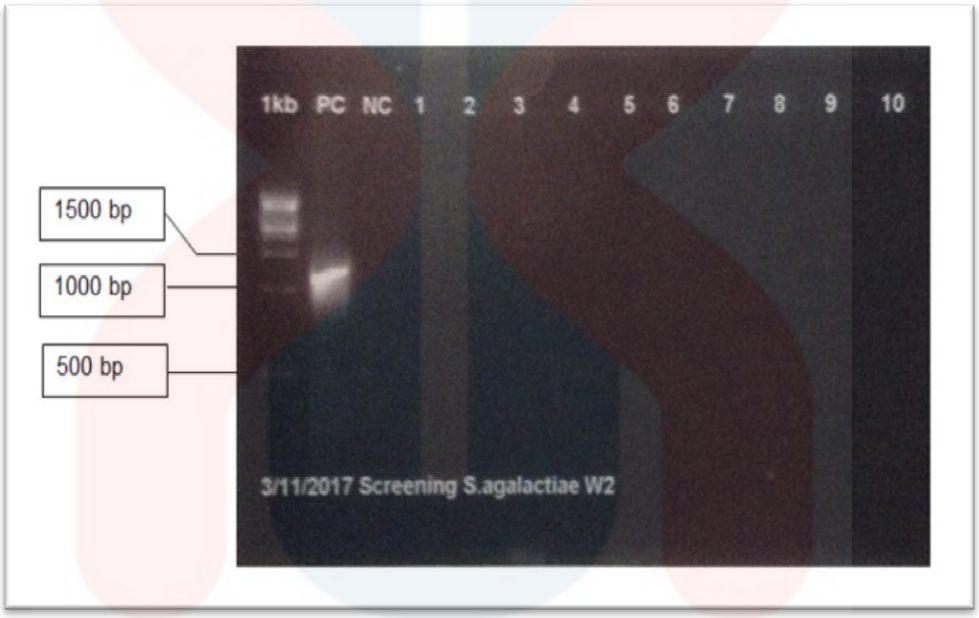

Figure 4.6: Visualization of PCR products for DNA sample of second sampling (2TAK), on agarose gel by electrophoresis. Molecular marker used was 1kb DNA ladder; PC: positive control (*S.agalactiae,* ATCC 13813); NC: negative control (DEPC water); lane 1-10: DNA fish isolates.

At the end of this study, a table was tabulated to determine the prevalence of the fish sample taken that is affected with *S.agalactiae.* It is found that none of the Tilapia that was sampled on all four sampling is affected with *S.agalactiae*. Thus, the prevalence for all four times of sampling is 0%.

Table 4.2: Prevalence of *S.agalactiae* throughout the sampling period.

Sampling ID	Positive PCR *S.agalactiae*	Prevalence (%)
1TAK	0/10	0
2TAK	0/10	0
3TAK	0/10	0
4TAK	0/10	0

CHAPTER 5

DISCUSSION

A total of 40 tilapia fish were samples and dissected to screen for the presence of *S.agalactiae*. Out of 40 samples, 5 fish was observed to have enlarged kidney during the necropsy. Other than that, all the internal and external observation of the fish showed no sign of abnormality.

Streptococcosis is a bacterial disease that affects fish and has caused severe economic loss in the production of freshwater and marine fish. Streptococcosis outbreak may occur at the same place or emerged from new area which was not reported with such disease due to improper farm management and poor biosecurity practice. Previous study has reported that *S. agalactiae* as one of the causative agent of Streptococcosis in tilapia (Thai Agricultural Standard, 2009).

Clinical sign of Streptococcosis like abnormal swimming can be detected in tilapia weighing between 100-300g which was reported more susceptible to *S. agalactiae* infection (Zamri-Saad et al., 2014). Supported by that fact, in this study the fish that was observed with clinical sign also have the same weight range. Gross sign of this disease includes exophthalmia, enlarge abdomen, hemorrhage and corneal opacity that can affect both eyes. For the internal lesion, the affected fish

shows presence of clear, viscous to hemorrhagic fluid in its abdominal cavity, pale and enlarge liver and kidney.

During the necropsy, there are some of the fish that observed with enlarged kidney but aside of that sign, overall appearance of the fish is normal. The enlarged kidney observed in the fish during the necropsy may due to a sign of early infection of the disease caused by other pathogen (Loch & Faisal, 2015).

After the necropsy, kidney tissue was collected from each fish and was then extracted using GF-1 Tissue DNA Extraction Kit. NanoDrop Spectrophotometer was used to checked for purity and quality of the extracted DNA. Reading from the NanoDrop showed that the DNA extracted has low quality after being compared to the general range of DNA purity level. Any abnormalities on 260/280 reading may be because of the contamination from the reagent that was used or phenol during the extraction procedure. While for 260/230 ratio, the low reading may resulted from the presence of unwanted organic contaminants (Desjardins & Conklin, 2010).

In this study, PCR was used to acquire a specific region of targeted DNA that is usually in between 150-3000 base pair (bp) in length (McPherson *et al.*,1991). In this study, the expected band desired for *S.agalactiae* was 1457 bp. The extracted DNA was subjected to PCR for amplifying the *S. agalactiae* specific 16S rDNA with forward primers 5′ GCT ATA CAT GCA GTA GAA CGC TGA 3′ and reverse primer 5′ ACG ACT TCA CCC CAA TCA TCT AT 3 (Amal et al., 2013). Primer used need to be long enough in order to allow a high annealing temperature and reduce the chance for nonspecific primer annealing to be occur, but if the primers is too long, it

may enable nonspecific annealing even to regions of DNA that are not perfectly complementary to the primer sequence.

16S rDNA is important for bacterial identification as it provided insights into etiologies of infectious disease. These genes are ideal targets for diagnosis for several reasons as they occur in tandem array along the chromosome and this multi-copy arrangement provides multiple targets for primer or probe binding (Waters and McCutchan, 1990). It also helps clinicians in choosing antibiotics and in determining the duration of treatment and infection control procedures (Woo, 2008). rDNA gene sequences are highly conserved within living organisms of the same genus and species, but that they differ between organisms of other genera and species (Cunningham, 2002).

Following the PCR optimization, band from lane five was chosen as a well-ned and brightest band. The selected band indicates the best annealing temperature at 52.8˚C. Well defined band is the most important characteristic compared to selecting brighter and thicker band which usually consist of multiple band or appropriate size of amplicons. This may be because of contamination of molecular grade water, the Taq polymerase or the primers with bacterial DNA (Breitschwerdt, 2003).

An optimal annealing temperature is important as it determine the end result of the PCR. If the annealing temperature in the specific reaction is too low, one or both primer would undergo non-specific annealing thus, leading to the amplification of undesired DNA sequence being targeted. The same goes to, when the annealing

temperature is too high, the possibility of primer annealing reduced, resulted on low DNA yield (Grunewald, 2007).

When the annealing temperature was optimized correctly, along with all other PCR components, the sensitivity of this method is possible to initiate single template molecule to replicate successfully. The optimized protocol used for the PCR was as follow; initial denaturation at 94˚C at 4 minutes, 35 cycles of denaturation at 94˚C at 30 seconds, annealing temperature of 52.8˚C for 30 seconds, extension at 72˚C for 1 minute followed by the final extension at 72˚C for 7 minutes. No expected band recorded as the PCR result showed negative sign of *S. agalactiae* in the DNA extracted.

All the samples obtained from sampling showed no sign of *S. agalactiae* except of enlarged kidney observed in some of the fish during necropsy. Aside from showing the clinical sign of usual Streptococcosis infection, the PCR results indicated no presence of *S.agalactiae* in the fish. The sign of enlarged kidney may indicates the early infection of other pathogen similar to *S.agalactiae*. As there is no positive PCR result of *S.agalactiae* on all the samples, therefore the prevalence of infection caused by *S.agalactiae* was 0%.

Stressful condition often leads to disease occurence. Therefore, it is essential to detect the pathogen from the carrier in order for an effective fish diseases control to happen. As years past, many great progressed have took place in molecular biology. It has become an important tool used to aid in diagnosing fish diseases and the epidemiology of the disease itself. Molecular techniques can be used to solve

these problems and increase the chance on detection of pathogen. These techniques include polymerase chain reaction (PCR), restriction enzyme digestion, probe hybridization, in situ hybridization, and microarray (Altinok & Kurt, 2004).

Meanwhile, as the advancement in technology has been grown rapidly, this study mainly focused on using the longest existing molecular technique which is PCR. As the result was negative for the entire sample, it may reflect to the farm management. Although the farm is quite small but the farmer has been managed it in a good way as majority of the fish in the pond was observed to be healthy. Besides, weather also can contribute to propagation of bacteria in fish that will eventually lead to disease infection. Bacteria is said to propagate actively during hot weather and become slow during cold and rainy weather (Amal & Zulkafli, 2013).

During PCR, there are so many problems that can cause inaccurate result. One of them is the choice of tissue sampled. In this study, only kidney tissue was taken from the fish. Various substances such as hemoglobin, bacterial constituents, and high concentrations of no target DNA can inhibit amplification (Wilson, 1997). In order to avoid contamination to occur during PCR, dissecting tools was used and changed for each fish or thoroughly clean after each use. A different room should be used for following steps of DNA extraction, PCR master mix preparation, DNA quantification and addition of DNA to PCR mixture, thermocycler and electrophoresis to avoid the possibility of cross contamination to occur between the samples.

CHAPTER 6

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Streptococcosis outbreaks in tilapia were reported in many countries, such as the United States of America, South Africa, Japan, Israel, Italy and Thailand (Thai Agricultural Standard, 2009). The disease was found in black and red tilapia, of all sizes. The disease occurrences were reported in all regions of tilapia producing countries.

This study was carried out with the aimed to screen the presence of *Streptococcus agalactiae* in tilapia fish (*Oreochromis sp.*) using molecular techniques and to determine the prevalence of the *S. agalactiae* infection from inland farm in Jeli, Kelantan. Based on the findings, it was observed that all the fish samples were free from *S.agalactiae* after the confirmation with PCR test. All 40 tilapia samples were cleared from any infection despite some of the fish having a sign of enlarged kidney. Thus, the study resulted in 0% prevalence of the *S.agalactiae* infection.

Based on negative PCR confirmation of *S. agalactiae* in all samples, it can be conclude that the farmer has been done a good job in keeping his farm properly managed and free from disease. This may be due to regular pond inspection done

every morning to observe the condition of the pond and its surrounding. It is done particularly during rainy season. By removal of weeds along the pond sides and clear the feeding platforms also can help prevent the occurrence of disease. All these action taken by the farmer indicates good farm management, thus making all the fish free from possible disease infection.

6.2 Recommendation

In order to get the desirable result from this study, some recommendation could be made into consideration. One of them is extend the period of the study. By extending the time period for the study to be carried out, accuracy of the data would be increase. Collect the data as much as possible. Next, carry out the sampling at more than one place. This would enable a comparison on each place to be made and more data could be collected at the same time. Prevalence also could be easily calculated with bigger amount of data.

Beside PCR, there are many other test and similar method that could be used to detect disease in fish. By conducting more than one test, the result would be more reliable and accurate. One of the tests is rapid staining test. The test could be carried out during post mortem examination. It could be done by collecting fresh tissue from skin or mucous membrane with pustules or inflammation, kidney or brain. They are then stained with Gram's stain and observed under light microscope. Furthermore, microbiological test also can be done in which it investigates disease at tissue level. Streptococcus bacteria are isolated and cultured from organs or tissues of infected

fish. Bacteria can be identified by testing the biochemical characteristics and bacterial morphology.

REFERENCES

Abdelhadi, Y. M. (2011). Tilapia : From the Nile to the World. *Journal of Agricultural Science and Technology*, *5*(2).

Alceste-Oliviero, C. (2000). Regional review of aquaculture status and development trends in the Latin America and Caribbean. Book of Synopses. NACA and FAO International Conference on Aquaculture in the Third Millenium, 20-25 February 2000, Bangkok, Thailand.

Altinok, I., & Kurt, I. (2004). Molecular Diagnosis of Fish Diseases : a Review. *Turkish Journal of Fisheries and Aquatic Sciences*, *138*(2003), 131–138.

Anon. (2014). How vaccines work. NPI reference guide on vaccine and vaccine safety. Retrieved from http://www.path.org/vaccineresources/files/ How_Vaccine_Work.pd.

Amal, M. N. A., Saad, M. Z., Siti Zahrah, A., & Zulkafli, A. R. (2013). Water quality influences the presence of Streptococcus agalactiae in cage cultured red hybrid tilapia, Oreochromis niloticus × Oreochromis mossambicus. *Aquaculture Research*, *46*(2), 1–11. https://doi.org/10.1111/are.12180

Amal, M. N. A., & Zulkafli, A. R. (2013). Molecular characterization of Streptococcus agalactiae strains isolated from fishes in Malaysia. https://doi.org/10.1111/jam.12210

Ang, K.J., Gopinath, N. and Chua, T. E. (1989). The status of introduced fish species in Malaysia, in S.S. de Silva (ed.) Exotic Aquatic Organism in Asia, Asian Fisheries Society Special Publication No. 3,71-82.

Baeck, G. W., Kim, J. H., Gomez, D. K., & Park, S. C. (2006). Veterinary Science Isolation and characterization of) in Jeju Island sp . from diseased flounder. *Journal of Veterinary Science*, *7*(March), 53–58. https://doi.org/10.4142/jvs.2006.7.1.53

Behrends, L. L., Nelson, R. G., Smitherman, R. 0., & Stone, N. M. (1982). Breeding and Culture of The Red -Gold Colour Phase of Tilapia. *Journal of World Mariculture Society*, *220*, 210–220.

Bernardet, J.F., Campbell, A.C. and Buswell, J.A. (1990). Flexibacter maritimus is the agent of 'black patch necrosis' in Dover sole in Scotland. Dis. Aquat. Org., 8: 233-237.

Breitschwerdt, E. B. (2003). Molecular diagnosis of infectious diseases: the good, the bad and the ugly, 1–6.

Chapman, F. A. (2015). Culture of Hybrid Tilapia : A Reference Profile, (August), 1–5. Retrieved from http://edis.ifas.ufl.edu/pdffiles/FA/FA01200.pdf

Cunningham, C. O. (2002). Molecular diagnosis of fish and shellfish diseases : present status and potential use in disease control, *206*, 19–55.

Desjardins, P., & Conklin, D. (2010). NanoDrop Microvolume Quantitation of Nucleic Acids 1 . Microvolume Nucleic Acid Quantification Using a NanoDrop 2000c Spectrophotometer 2 . High-Sensitivity Microvolume Nucleic Acid Quantitation Using the NanoDrop 3300, (November), 1–4. https://doi.org/10.3791/2565

Evans, J.J., Klesius, P.H., Gilbert, P.M., Shoemaker, C.A., Al Sarawi, M.A., Landsberg, J., Duremdez R., Al Marzouk A. and Al Zenki S. (2002),Characterization of b-hemolytic group B Streptococcus agalactiae in cultured sea bream, Sparus auratus L., and wild mullet, Liza klunzingeri, in Kuwait. Journal of Fish Diseases, 25, 505–513

FAO. (2010). National Aquaculture Sector Overview. Retrieved from http://www.fao.org/figis/pdf/fishery/countrysector/naso_malaysia/en?title=FAO Fisheries %26 Aquaculture - National Aquaculture Sector Overview - Malaysia

Garibyan, L., & Avashia, and N. (2013). Polymerase Chain Reaction. *Genomics*, *133*(3), 1–6. https://doi.org/10.1038/jid.2013.1

Grunewald, H. (2007). *Optimization of Polymerase Chain Reaction*. (J. M. . Bartlett & D. Stirling, Eds.). Totowa, New Jersey: Humana Press Inc. https://doi.org/10.1385/1-59259-384-4:89

Gupta, M. V, & Acosta, B. O. (2004). A review of global tilapia farming practices, *IX*(1).

Inglis, V., Robert, R.J., & Bromage, N.R. (1993). Bacterial disease of fish (pp. 196-207). London: Blackwell Science Ltd.

Kechik, I. A. (1995). Aquaculture in Malaysia. *SEAFDEC Aquaculture Department. Kechik IA. Aquaculture in Malaysia*, (1995), 125–135. Retrieved from http://hdl.handle.net/10862/113

Kechik, I. A. (2015). Aquaculture in Malaysia Aquaculture in Malaysia, (1995).

Kim, J.H., Gomez, D.K., Choresca, C.H., & Park, S.C. (2007). Detection of major bacterial and viral pathogens in trash fish used to feed cultured flounder in Korea. Aquaculture, 272, 105-110.

Klesius, P.H., Shoemaker, C.A., & Evans, J.J. (2008). Streptococcus: A worldwide fish health problem. 8th International Symposium on Tilapia in Aquaculture (pp. 83-107). Cairo

Loch, T. P., & Faisal, M. (2015). Emerging flavobacterial infections in fish : A review. *Journal of Advanced Research*, *6*(3), 283–300. https://doi.org/10.1016/j.jare.2014.10.009

McPherson, M.J., Quirke, P., Taylor, G.R., (1991). PCR: A Practical Approach. Oxford Univ. Press, Oxford, 253 pp

Mullis, K. (1990). The Unusual Origin of the Polymerase Chain Reaction. Scientific American.

Musa, N., Seong Wei, L., Musa, N., Hamdan, R. H., Kok Leong, L., Wee, W., Abdullah, S. Z. (2009). Streptococcosis in red hybrid tilapia (Oreochromis niloticus) commercial farms in Malaysia. *Aquaculture Research*, *40*(5), 630–632. https://doi.org/10.1111/j.1365-2109.2008.02142.x

Ndong D., Chen Y.Y., Lin Y.H., Vaseeharan B. & Chen J.C. (2007) The immune response of Mozambique tilapia Oreochromis mossambicus (Peters) and its susceptibility to Streptococcus iniae under stress in low and high temperatures. Fish and Shellfish Immunology 22, 686–694.

Nguyen, H.T., Kanai, K., & Yoshikoshi, K. (2002). Ecological investigation of S. iniae isolated in cultured Japanese Flouder, Paralicthys olivaceus using selective isolation procedure. Aquaculture, 205, 7-17.

Pazos, F., Santos, Y., Macías, A.R., Núñez, S. and Toranzo, A.E. (1996). Evaluation of media for the successful culture of Flexibacter maritimus. J. Fish Dis., 19: 193-197.

Pongthana, N., Nguyen, N. H., & Ponzoni, R. W. (2010). Comparative performance of four red tilapia strains and their crosses in fresh- and saline water environments. *Journal of Aquaculture*, *308*(SUPPL.1), S109–S114. https://doi.org/10.1016/j.aquaculture.2010.07.033

Siti-Zahrah, A., Padilah, B., Azila, A., Rimatulhana, R., & Shahidan, H. (2008). Multiple Streptococcal Species Infection in Cage-cultured Red Tilapia But Showing Similar Clinical Signs, 313–320.

Public Health England. (2014)Identification of Streptococcus species, Enterococcus species and Morphologically Similar Organisms. (pp. 1–36). England: Standard Unit, Public Health England.

Smith, C., Osborn, M. (2009) Advantages and limitations of quantitative PCR (qPCR)-based approaches in microbial ecology. FEMS Microbiol Ecol 67:6–20

Sun, J., Fang, W., Ke, B., He, D., Liang, Y., Ning, D., Tan, H., Peng, H., Wang, Y., Ma, Y., Ke, C., Deng, X. (2016). Inapparent Streptococcus agalactiae infection in adult/commercial tilapia. *Scientific Reports*, *6*, 26319. https://doi.org/10.1038/srep26319

Thai Agricultural Standard. (2009). *National Bureau of Agricultural Commodity and Food Standards National Bureau of Agricultural Commodity and Food Standards*.

Vogel J, Yee C, Darling J (2012) Molecular biology. In: Bolognia J, Jorizzo J, Rapini R (eds) Dermatology, 3rd edn. Elsevier: Philadelphia, PA, 65–79

Watanabe, W. O., Losordo, T. M., Fitzsimmons, K., & Hanley, F. (2002). *Tilapia Production Systems in the Americas : Technological Advances , Trends , and Challenges*. London, UK: Taylor & Francis. https://doi.org/10.1080/20026491051758

Waters, A.P., McCutchan, T.F., (1990). Ribosomal RNA: nature's own polymerase-amplified target for diagnosis. Parasitol. Today 6, 56–59.

William W. Wilfinger, Karol Mackey, and Piotr Chomczynski. (1997). Effect of pH and Ionic Strength on the Spectrophotometric. Assessment of Nucleic Acid Purity: BioTechniques 22:474-481

Wing-Keong, N., Sih-Win, T., Kabir, M. A., Chowdhury, & Bureau, D. P. (2013). On-farm feeding and feed management in aquaculture. *FAO Fisheries and Aquaculture Technical Paper No. 583. Rome, FAO.*, 407–431.

Woo, P. C. Y., Lau, S. K. ., Teng, J. L. ., Tse, H., & Yuen, K. (2008). *Then and now : use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories*.

Zamri-Saad, M., Amal, M. N. A., Siti-Zahrah, A., & Zulkafli, A. R . (2014). Control and prevention of streptococcosis in cultured tilapia in malaysia: A review. *Pertanika Journal of Tropical Agricultural Science*, *37*(4), 389–410.

APPENDIX

Table A. 1: Sampling of fish on 24/09/2017 (Week 1).

Code	Weight (g)	External observation	Internal observation	PCR
1TAK1	152.50	Normal skin pigmentation, eyes and fin	Normal	Negative
1TAK2	125.30	Normal skin pigmentation, eyes and fin	Normal	Negative
1TAK3	137.50	Normal skin pigmentation, eyes and fin	Enlarged kidney	Negative
1TAK4	145.00	Normal skin pigmentation, eyes and fin	Normal	Negative
1TAK5	150.70	Normal skin pigmentation, eyes and fin	Normal	Negative
1TAK6	173.30	Normal skin pigmentation, eyes and fin	Normal	Negative
1TAK7	145.70	Normal skin pigmentation, eyes and fin	Normal	Negative
1TAK8	155.43	Normal skin pigmentation, eyes and fin	Normal	Negative
1TAK9	149.33	Normal skin pigmentation, eyes and fin	Enlarged kidney	Negative
1TAK10	164.75	Normal skin pigmentation, eyes and fin	Normal	Negative

Table A. 2: Sampling of fish on 11/10/2017 (Week 2).

Code	Weight (g)	External observation	Internal observation	PCR
2TAK1	142.37	Normal skin pigmentation, eyes and fin	Normal	Negative
2TAK2	150.00	Normal skin pigmentation, eyes and fin	Normal	Negative
2TAK3	169.30	Normal skin pigmentation, eyes and fin	Normal	Negative
2TAK4	130.75	Normal skin pigmentation, eyes and fin	Normal	Negative
2TAK5	144.00	Normal skin pigmentation, eyes and fin	Enlarged kidney	Negative
2TAK6	165.43	Normal skin pigmentation, eyes and fin	Normal	Negative
2TAK7	137.90	Normal skin pigmentation, eyes and fin	Normal	Negative
2TAK8	159.60	Normal skin pigmentation, eyes and fin	Normal	Negative
2TAK9	151.20	Normal skin pigmentation, eyes and fin	Normal	Negative
2TAK10	130.75	Normal skin pigmentation, eyes and fin	Enlarged kidney	Negative

Table A. 3: Sampling of fish on 26/10/2017 (Week 3).

Code	Weight (g)	External observation	Internal observation	PCR
3TAK1	134.65	Normal skin pigmentation, eyes and fin	Normal	Negative
3TAK2	150.03	Normal skin pigmentation, eyes and fin	Normal	Negative
3TAK3	129.53	Normal skin pigmentation, eyes and fin	Normal	Negative
3TAK4	144.00	Normal skin pigmentation, eyes and fin	Normal	Negative
3TAK5	153.33	Normal skin pigmentation, eyes and fin	Normal	Negative
3TAK6	123.45	Normal skin pigmentation, eyes and fin	Normal	Negative
3TAK7	137.95	Normal skin pigmentation, eyes and fin	Normal	Negative
3TAK8	137.41	Normal skin pigmentation, eyes and fin	Normal	Negative
3TAK9	165.32	Normal skin pigmentation, eyes and fin	Enlarged kidney	Negative
3TAK10	143.70	Normal skin pigmentation, eyes and fin	Normal	Negative

Table A. 4: Sampling of fish on 9/11/2017 (Week 4).

Code	Weight (g)	External observation	Internal observation	PCR
4TAK1	147.93	Normal skin pigmentation, eyes and fin	Normal	Negative
4TAK2	137.00	Normal skin pigmentation, eyes and fin	Normal	Negative
4TAK3	159.75	Normal skin pigmentation, eyes and fin	Normal	Negative
4TAK4	117.49	Normal skin pigmentation, eyes and fin	Normal	Negative
4TAK5	149.23	Normal skin pigmentation, eyes and fin	Normal	Negative
4TAK6	151.36	Normal skin pigmentation, eyes and fin	Normal	Negative
4TAK7	143.15	Normal skin pigmentation, eyes and fin	Normal	Negative
4TAK8	133.77	Normal skin pigmentation, eyes and fin	Normal	Negative
4TAK9	131.39	Normal skin pigmentation, eyes and fin	Normal	Negative
4TAK10	145.54	Normal skin pigmentation, eyes and fin	Enlarged kidney	Negative

Table A. 5: Mean and Standard error on weight of samples.

Sampling	Mean Body Weight (g)	Standard Error (SE)
Sampling 1 (1TAK)	149.951	±4.22
Sampling 2 (2TAK)	147.13	±4.28
Sampling 3 (3TAK)	141.937	±3.85
Sampling 4 (4TAK)	141.661	±3.81

Table A. 6: Nanodrop reading on DNA sample of Week 1.

Code	**Organ**	**(DNA/ng)**	**(260/280)**	**(260/230)**
1TAK1	Kidney	62.9	1.87	3.16
1TAK2	Kidney	24.1	1.70	3.90
1TAK3	Kidney	38.8	1.84	3.64
1TAK4	Kidney	53.4	1.78	3.58
1TAK5	Kidney	1.5	1.02	-0.29
1TAK6	Kidney	8.1	1.75	-1.89
1TAK7	Kidney	3.6	1.17	-2.03
1TAK8	Kidney	10.5	1.86	13.40
1TAK9	Kidney	6.0	2.17	3.44
1TAK10	Kidney	8.4	1.42	-1.05

Table A. 7: Nanodrop reading on DNA sample of Week 2.

Code	**Organ**	**(DNA/ng)**	**(260/280)**	**(260/230)**
2TAK1	Kidney	30.3	1.94	2.80
2TAK2	Kidney	54.4	1.92	2.94
2TAK3	Kidney	56.7	1.85	2.90
2TAK4	Kidney	42.2	1.91	2.39
2TAK5	Kidney	79.0	1.88	2.78
2TAK6	Kidney	62.1	1.88	2.78
2TAK7	Kidney	63.0	1.92	2.89
2TAK8	Kidney	16.4	1.81	4.86
2TAK9	Kidney	50.5	1.86	2.78
2TAK10	Kidney	11.6	1.79	12.53

Table A. 8: Nanodrop reading on DNA sample of Week 3.

Code	Organ	(DNA/ng)	(260/280)	(260/230)
3TAK1	Kidney	23.0	1.75	2.43
3TAK2	Kidney	26.3	1.98	2.66
3TAK3	Kidney	17.3	1.84	3.69
3TAK4	Kidney	34.1	1.85	3.07
3TAK5	Kidney	174.4	1.92	2.57
3TAK6	Kidney	161.7	1.89	2.43
3TAK7	Kidney	31.0	1.94	3.16
3TAK8	Kidney	83.4	1.94	2.53
3TAK9	Kidney	107.6	1.93	2.42
3TAK10	Kidney	26.6	1.84	1.52

Table A. 9: Nanodrop reading on DNA sample of Week 4.

Code	Organ	(DNA/ng)	(260/280)	(260/230)
4TAK1	Kidney	13.2	1.65	2.66
4TAK2	Kidney	30.3	1.98	2.49
4TAK3	Kidney	20.3	1.64	2.37
4TAK4	Kidney	35.9	1.65	3.19
4TAK5	Kidney	57.4	1.74	2.63
4TAK6	Kidney	16.7	1.62	2.54
4TAK7	Kidney	40.0	1.78	2.86
4TAK8	Kidney	12.8	1.78	2.43
4TAK9	Kidney	17.6	1.60	3.42
4TAK10	Kidney	26.6	1.79	1.30