Molecular Screening on The Existence of *Edwardsiella tarda* in Hybrid Red Tilapia (*Oreochromis sp*.) From Inland Farm in Jeli, Kelantan

By

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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.

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Supervisor

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Molecular Screening on The Existence of *Edwardsiella tarda* in Hybrid Red Tilapia (*Oreochromis sp*.) From Inland Farm in Jeli, Kelantan

ABSTRACT

Edwardsiella tarda is an emerging fish pathogen that jeopardize the aquaculture industry worldwide by causing significance economic loss. Infection by *E.tarda* can be characterized by symptoms and clinical signs that varies depending on the aquaculture species. Lack of knowledge among farmers in Kelantan on edwardsiellosis induce risk on the public health of consumer. The present study aimed to screen for *E.tarda* infection in hybrid red tilapia from inland farm in Jeli through molecular screening hence determined the prevalence of *E.tarda* infection. Forty hybrid red tilapia weighed between 141.66 ± 4.02 g and 150.73 ± 3.71 g were randomly collected twice a month throughout two months of sampling period. Any abnormalities during necropsy of the fish sampled was recorded. The DNA was extracted from fish kidney and subjected to molecular screening through conventional polymerase chain reaction (PCR). Optimised PCR protocol was used for the rest of amplification reaction with annealing temperature at 52.8°C. The presence of *E.tarda* through PCR was confirmed through expected amplicon size of 415bp. From the result obtained, enlargement of kidney was observed during necropsy of sampled fish which indication to one of *E.tarda* infection clinical sign. On top of that, prevalence of *E.tarda* was the highest in Week 1 which contributed 40% prevalence while for Week 2 until Week 4, no prevalence was recorded. Even though there was low prevalence of *E. tarda* detected in the samples, it still may lead to outbreak of disease in the farm. Thus, development of prevention method against this pathogen deserved a serious attention and appropriate control need to be considered.

Keywords: *Edwardsiella tarda,* Polymerase chain reaction (PCR), hybrid red tilapia, edwardsiellosis, kidney

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Penyaringan Molekul ke atas Kewujudan *Edwardsiella tarda* dalam Tilapia Merah Hibrid (*Oreochromis sp.)* Dari Ladang Daratan di Jeli, Kelantan

ABSTRAK

Edwardsiella tarda adalah vybridvn ikan yang baru muncul yang menjejaskan vybridvn akuakultur di seluruh dunia dengan mengakibatkan kerugian ekonomi yang ketara. Jangkitan oleh *E.tarda* boleh dikategorikan dengan pelbagai gejala dan tanda klinikal yang berbeza-beza bergantung kepada spesies akuakultur. Kekurangan ilmu mengenai edwardsiellosis di kalangan penternak di Kelantan mendorong risiko kepada kesihatan masyarakat pengguna. Matlamat kajian ini adalah untuk menyaring jangkitan *E.tarda* dalam tilapia merah vybrid dari ladang daratan di Jeli melalui saringan molekul seterusnya menentukan kadar prevalens jangkitan *E.tarda*. Empat puluh ekor tilapia merah vybrid dengan berat antara 141.66 ± 4.02 g dan 150.73 ± 3.71 g telah diambil secara rawak dua kali sebulan sepanjang dua bulan tempoh persampelan. Sebarang keabnormalan semasa nekropsi ikan telah dicatatkan. Seterusnya, DNA dari buah pinggang ikan telah diekstrak dan tertakluk kepada penyaringan molekul melalui tindak balas rantaian polimerase (PCR) konvensional. Protokol PCR yang telah dioptimumkan digunakan dalam tindakbalas amplifikasi yang berbaki dengan suhu penyepuhlindapan pada 52.8°C. Kewujudan *E.tarda* melalui PCR telah disahkan dengan jangkaan vybridvn bersaiz 415bp. Daripada hasil kajian yang diperoleh, pembesaran buah pinggang dapat dicerap semasa nekropsi sampel ikan yang menunjukkan salah satu tanda klinikal berkaitan dengan jangkitan *E.tarda*. Tambahan pula, prevalens *E.tarda* adalah yang tertinggi dalam Minggu 1 yang menyumbang kepada 40% prevalens sementara untuk Minggu 2 sehingga Minggu 4, tiada prevalens yang direkodkan. Walaupun prevalens jangkitan *E.tarda* yang dikesan dari sampel adalah rendah, ia masih boleh menyebabkan wabak penyakit di dalam ladang. Oleh itu, pembangunan kaedah pencegahan terhadap vybridvn ini layak mendapat perhatian yang serius dan kawalan yang bersesuaian perlu dipertimbangkan.

Kata kunci: *Edwardsiella tarda,* tindak balas rantaian polimerase, tilapia merah vybrid, edwardsiellosis, buah pinggang

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LIST OF ABBREVIATIONS AND SYMBOLS

PCR	Polymerase chain reaction
RAPD	Random Amplified Polymorphic DNA
TSA	Trypton soy agar
ET	*Edwardsiella tarda* agar
BHIA	Brain heart infusion agar
EIM	Edwardsiella isolation media
TSB	Tryptic soy broth
SS	Salmonella-Shigella agar
MA	Marine agar
XLD	Xylose lysine media
DEPC	Diethyl pyrocarbonate
TBE	Tris-borate EDTA
DNA	Deoxyribonucleic acid
RPS	Relative percentage survival
ATCC	American Type Culture Collection
$MgCl_2$	Magnesium chloride
dNTPs	Deoxynucleotide triphosphate
UV	Ultra violet
UMK	Universiti Malaysia Kelantan
°C	Degree Celsius
µl	Microliter
%	Percentage
g	Grams
mg	Milligrams

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µm	Micrometre
ng	Nanograms
bp	Base pair
kb	Kilo base
n	Number

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Broadly, the term aquaculture can be interpreted as aquafarming that focused on activity such as breeding, rearing and harvesting of aquatic organism (FAO, 1987). Aquatic organisms that involve in aquaculture activity including fish, crustacean, mollusk and aquatic plant that reared in different type of water environments. The earliest aquatic farming activity was recorded in China that mainly utilized the common carp, *Cyprinus carpi.* Since that, the aquaculture husbandry has developed phase by phase in China and has influenced the development of aquaculture industry in other countries. Aquaculture industry continued to grow widely due to high demand in fish protein and it is reported that Asia has become the main contributors of the world's aquaculture production (Lee & Wendy, 2014).

In Malaysia, the aquaculture industry is one of the main sub sectors under fisheries and it contributes about 16% of seafood supply while the major contributor for fisheries sector is marine capture fisheries industry which contribute about 84% of total seafood supply (Ng, Teh, Chowdhury & Bureau, 2013).

Nile tilapia or known as *Oreochromis niloticu*s is the most common and important freshwater fish species cultured worldwide. History of tilapia in Malaysia began in 1952 when Mozambique tilapia, *O*. *mossambicus*, was the first species introduced to farmers where they used the earthen pond and in ex mining pool to culture this species. Later, in 1980's, the hybrid red tilapia, *Oreochromis sp.,* was introduced in Malaysia as the commercial tilapia aquaculture resulting from the crossbreed between Nile tilapia (*O. niloticus)* and Mozambique tilapia (*O. mossambicus).* The commercial hybrid red tilapia were cultured using improved culture system such concrete tanks, floating cage and earthen ponds (Hashim, 2015).

Since 1980's , the hybrid red tilapia production undergo significant growth and become the main contributor of total tilapia production in which contributes 82% production followed by Nile tilapia (*O. niloticus)* and Mozambique tilapia (*O. mossambicus)* (Ng et.al., 2013).

As the aquaculture activity become widely expand and intensify, disease outbreak in fish culture system becoming more uncontrollable hence marked disease as the significant obstacle to the aquaculture industry. Disease occurs when there is an interaction among pathogen, host and environment (Caipang, Jomer & Clara, 2014).

Tilapia can be infected by infectious and non-infectious disease and the critical season in Malaysia is during drought season where the total production can reduced to half due to occurrence of mass mortality. The most reported disease that attacks fish culture is bacterial disease followed by viruses, parasites and fungi (Lee & Wendy, 2014). Bacteria that commonly found in tilapia cultured are *Streptococcus spp.,*

Flavobacterium, Aeromonas hydrophila, Mycobacterium fortuitous and *Edwardsiella tarda* (Lee & Wendy, 2014).

In recent year, there is an emerging bacterium pathogen known as *Edwardsiella tarda (E.tarda)* that responsible for edwardsiellosis disease in fish. Outbreak of edwardsiellosis disease has been reported in Japan, China, Europe, Korean, Venezuela and Greece (Kusuda & Kawai, 1998; Clavijo, Conroy, Conroy, Santander & Aponte, 2002; Alcaide, Herraiz & Esteve, 2006; Yu, Han, Park, Park & Park, 2009; Kokkari, Dourala, Kalatzis, Smyrli, Jun, & Katharios, 2014; Mo, Zhou, Zhang, Gan, Liu & Dan, 2015).

1.2 Problem Statement

In Malaysia, there are many small holder of fish farming especially in the rural area and some of the farmers are lack in knowledge of disease management. Due to the increasing demands from the consumer around them, their fish farming activity becoming more intensify such as overcrowding which deteriorate the aquatic environment hence increase the fish susceptibility towards pathogen. Thus, outbreaks of disease occur more frequently.

Fish pathogen such as *E.tarda* hold the potential to become one of the most important pathogen in fish culture and towards human health. Lack of knowledge on this bacteria among the farmers can harm consumer as well to the fish farmed. Serious infection of edwardsiellosis increase fish mortalities in the fish population and also induce adverse effect on consumer health by contacting or consuming infected fish.

When the fish farmed showing symptom of disease, most of the farmer will harvest their fish because they want to avoid economic loss. Instead of investigating the cause of the situation, they prefer to sell to the consumer without knowing the risk of infection that their fish carry.

1.3 Objectives

1. To screen the presence of *E.tarda* in hybrid red tilapia from inland farm in Jeli, Kelantan through molecular technique.
2. To determine the prevalence of *E.tarda* in hybrid red tilapia from inland farm in Jeli, Kelantan.

1.4 Scopes of Study

The scopes of study involved are aquatic animal health and molecular biology.

1.5 Significance of Study

Infection of *E. tarda* lead to edwardsiellosis outbreak in fish population either in marine or freshwater environment. *Edwardsiella tarda* has trigger the aquaculture industry awareness due to its ability to cause disease not only in aquatic organism also in other animals and humans. The prevalence of edwardsiellosis incidence also has been reported increasing worldwide. There are many studies have been done to investigate the pathogenicity and virulency of *E.tarda* in order to understand this bacterium.

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Edwardsiellosis outbreak in fish farm were reported in previous studies has caused high mortality from 10% to 90% in young tilapia and 100% mortality within 30 days in Japanese flounder. The used of commercial antibiotic has no longer effective to prevent this infection. One of the factors that lead to rapid transmission in fish population is the handling of infected fish such as during harvesting and transferring into overcrowding holding tanks. Clinical signs that appeared due to this infection can be confused with other bacterial infection such as exophthalmia, enlargement of internal organ and hemorrhages. Hence, molecular technique is a method used to confirm the *E.tarda* infection.

In Malaysia, this bacteria has been isolated from several aquatic species for such as Asian seabass and giant freshwater prawn. Thus, the aim of this study were to screen the presence of *E.tarda* through molecular technique and followed by determination of *E.tarda* prevalence in hybrid red tilapia from inland farm in Kelantan. The prevalence obtained from this study can be used to warn farmers to take early prevention before the edwardsiellosis outbreak take place in their farm. Besides, it will help the farmers to control the presence of *E.tarda* from spreading to nearby fish pond.

Furthermore, the findings of this study were able to expand knowledge and update current status on bacterial disease affecting tilapia cultured in Kelantan. Last but not least, the *E.tarda* prevalence obtained from this study can be used as future reference

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CHAPTER 2

LITERATURE REVIEW

2.1 Hybrid Red Tilapia (*Oreochromis sp*.)

Tilapia is an important commercial cultured fish species belonging to the family of Cichlids and consist of three genera which are *Sarotherodon*, *Oreochromis* and *Tilapia*. These three genera are distinguished by the way of parental care provide to their young (Chapman, 2000).

For the mouth brooding genera *Sarotherodon* and *Oreochromis*, the parents will incubate the young in their mouth in order to protect them. However, fish species in genus *Oreochromis* is slightly differ from the genus *Sarotherodon* in a way that female is the one that primarily perform the parental care towards their young. On the other hand, species in the genus *Tilapia* will incubate their egg in a nest substrate build at the bottom of the pond or lake (Chapman, 2000).

Nowadays, there are many tilapia species exist that come from the native species and also cross breed between species to produce a better hybrid tilapia for example Mozambique tilapia or also known as Java tilapia (*O.mossambicus*, blue tilapia (*O.aureus*), Nile tilapia (*O. niloticus*) and hybrid red tilapia. Originally, the distribution of tilapia only native to Israel, Jordan and Africa somehow the distribution

has increasing worldwide hence becoming one of the most important cultured fish (Lovshin, 2000).

Among of all the species of tilapia, Nile tilapia is the most cultured in farm due to their favourable characteristic such as rapid growth rate, late age of sexual maturity and feed on plankton habits (Lovshin, 2000). Recently, the hybrid red tilapia has gained popularity among the farmers to replace the Nile tilapia due to the attractive characteristics such as red colour which increase the marketability and also high resistance towards salinity. Hybrid red tilapia can be cultured either in fresh water or marine water due to this characteristic.

Hybrid red tilapia is resulted from the selective breeding programs and cross of selected species in the genus *Oreochromis* has developed several strain of red tilapia. Taiwanese red tilapia was originally reported for the first time in Taiwan during the late 1960s and was produced from a cross between a normal male Nile tilapia and a mutant reddish orange female Mozambique tilapia. It was the first strain of hybrid red tilapia produced and later, new red hybrid strain was produced in Florida. Florida red tilapia was the cross between a red gold male of Mozambique tilapia and female of normal Zanzibar tilapia (*O. hornorum*) (Lovshin, 2000).

The cross between *Oreochromis* species continued in Israel where the third strain of red tilapia was produced. In Malaysia, tilapia broodstock was being imported from private hatcheries in country such as Thailand and Taiwan. Hence, multiple breeding program for example cross breeding and hybridization were done to improve the tilapia strain including producing new hybrid. These breeding program will enhance

the performance of each strain and increase the essential characteristic such as growth and survival rate, appearance of the fish including colour and body weight (Hamzah, Nguyen, Ponzoni, Kamaruzzaman & Subha, 2008).

In Malaysia, hybrid red tilapia is the dominant species of fresh water fish being farm and contributes 82% of total tilapia production. The advantages of culturing this hybrid red tilapia compared to other species of tilapia is higher consumer acceptance due to its color. Besides, hybrid red tilapia is suitable to be raised in brackish water and sea water because of the salinity tolerance. The common culture system used to grow tilapia for instance in cages, tanks, ponds and also large water bodies including reservoirs and former mining pools (Hamzah et al., 2008).

Due to the rapid growth of aquaculture activity, the prevalence of disease outbreaks become more intense. For that reason, there are several diseases that mark as most threatening disease infection in tilapia farming such as Streptococcosis and Vibriosis. Hybrid red tilapia is well known for its high resistance to disease but due to favorable factors that allow pathogen to grow in the culture environment for instance, deterioration of water quality and temperature change, may increase the susceptibility of hybrid red tilapia to get infected.

Streptococcosis is one of the most important bacterial diseases affecting tilapia farming worldwide. The diseases commonly occur due to the infection of *Streptococcus inaie* and *S. agalactiae.* Due to the global distribution of *S.inaie* and *S.agalactiae,* there are about 27 species of fish including tilapia that are susceptible to this disease (Agnew & Barnes, 2007). Clinical signs that can be observed from the infected tilapia including

hemorrhage at the gill, eye and the base of the fins. Moreover, internal organ such as spleen and liver appeared to be enlarged (Amal & Zamri- Saad, 2001).

Beside *Streptococcus spp*., *Vibrio spp*. is also known as fish pathogens that pose a serious threat to tilapia farming. *Vibrio spp*. is responsible for Vibriosis disease that usually occur during the early stage of larval. Common clinical signs that can be observed include exophthalmia, hemorrhaging in the mouth, damaged eye and ulcers (Al-Sunaiher, Ibrahim & Al-Salamah, 2010).

Apart from the above disease, there is notable emerging pathogen that has rise the aquaculture industry awareness on their ability to cause disease. The emerging pathogens are *Francisella spp*. and *E. tarda.* Other than bacteria, there are also fungal and virus infection that posed threat to the tilapia farming worldwide. Current researches and studies have developed many effective vaccines to treat and control the disease outbreak hence can avoid economic loss.

2.2 *Edwardsiella tarda*

Edwardsiella tarda is an emergent bacterial pathogen that has been known to cause severe disease outbreak named edwardsiellosis along with serious economic loss in both marine and freshwater aquaculture as well as in wild fish population. Emergent pathogen can be manifested as newly discovered or new strains that arise from existing pathogen hence increase the incidence of epidemic outbreak to occur (Woolhouse & Dye, 2001).

Initially, *E.tarda* was known as *Paracolobactrum anguillimortiferum*, an organism that described as fish pathogen by Hoshina (Bullock & Herman, 1985). However, due to the loss of original culture of *P. anguillimortiferum* and it was not registered, *E.tarda* was designated by Ewing, McWhorter, Escobar & Lubin (1965) to replace the *P. anguillimortiferum* and since that *E.tarda* has been accepted worldwide (Alcaide et al., 2006; Garcia, Iregui & Hirono, 2012; Park, Aoki & Jung, 2012).

Edwardsiella genus within Enterobacteriaceae family has 3 different species known as *E.tarda*, *E. ictaluri* and *E. hoshinae. Edwardsiella ictaluri* which has DNA similarity about 56%-62% to *E.tarda* is well known as an important causative agent of enteric septicemia disease in catfish and is considered as host-specific pathogen of catfish (Hawke et al., 2013). On the other hand, *E.tarda* is known to have the broadest range of host distribution where it can infect both warm water and cold water fish species, amphibians, reptiles, warm blooded mammals including birds and humans. Moreover, *E. hoshinae* is less found in the nature and usually infect birds and reptiles (Joh et al., 2011; Park et al., 2012; Leung, Siame, Tenkirk, Noort & Mok, 2012).

Apart from aquatic species, *E.tarda* also is a health threat to other animal species. This bacterium has been recognised to cause an extraintestinal infection and intestinal infection in amphibian, reptiles, birds and mammals. Some of the animal may exhibit concomitant clinical sign that may be caused by the natural bacterial in the digestive tract or state of being a carrier for *E.tarda* (Garcia et al., 2012).

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Edwardsiella tarda infection in humans can occur in two different way. Firstly, *E.tarda* cause intestinal disease which can be characterized by acute watery diarrhea and vomiting. Once the bacteria has been introduced into the gastrointestinal tract, the infection occur and it appear to be similar to Salmonella gastroenteritis condition. The occurrence of intestinal disease cause by *E.tarda* is more frequent in tropical and sub-tropical region due to the dietary habits consumer in these region where they are more likely to consume raw meat of fish and seafood (Wang et al., 2005; Garcia et al., 2012).

On the other hand, *E.tarda* can also cause extraintestinal disease that occur due to wound obtained during fishing, diving or abdominal trauma. Hence, the bacteria escape from the bowel and propagate into adjacent tissues. There was case of *E.tarda* extraintestinal infection that have been reported in Taiwan. Even though extraintestinal disease in humans are rarely reported, but it may cause mortality to human. Especially in the case where the pregnant woman is infected with extraintestinal disease, the bacteria will colonize the maternal birth canal and will transmit to the foetus. Later during labour, it will cause neonatal infection to the new born which mean the new born may die during the first 28 days of life. There was a case report related to neonatal infection in Nigeria where the mother consumed snake flesh and it became the source of infection (Mowbray, Buck, Humbaugh & Marshall, 2003).

2.2.1 Characteristic of *E.tarda*

Edwardsiella tarda is described as a Gram-negative, short, straight rod–shaped, facultative anaerobic bacterium which means the bacteria can grow and survive with the presence or absence of the oxygen. The length of the *E.tarda* is about 2-3 µm and has diameter about 0.5 µm to 1 µm. Most of the time, *E.tarda* can be

considered as motile by peritrichous flagella except for the bacteria that isolate from the red sea bream and yellowtail.

Edwardsiella tarda can be isolated and cultured on differential and selective media such as trypton soy agar (TSA), MacConkey agar, marine agar (MA), Xylose Lysine Deoxycholate (XLD), *Edwardsiella tarda* agar (ET) Brain Heart Infusion agar (BHIA), Edwardsiella Isolation Media (EIM), Rimler-Shotts selected media and tryptic soy broth (TSB), Salmonella–Shigella agar (SS) (Castro, Toranzo, Nunez & Magarinos, 2011; Kebede & Habtamu, 2016).

2.2.2 Occurrence of *E.tarda*

Health status of fish in farm culture system is challenging to control and maintain as any extreme changes or deterioration of aquatic environment resulting in disease occurrence in fish population. High water temperature, poor water quality, overstocking as well as high organic contain in the aquatic environment may lead to the occurrence of edwardsiellosis in fish.

Until this day, *E.tarda* has been reported to infect various economically important fish species worldwide. List of fish species that has been infected with *E.tarda* including Japanese eel (*Anguilla japonica*), channel catfish (Ictaluri *punctatus*), striped bass (Morone *saxatili*), European eel (Anguilla *anguilla*), tilapia *(Oreochromis niloticus*), Japanese flounder (*Paralichthys olivaceus*), yellow catfish (*Pelteobagrus fulvidraco*), red sea bream (*Pagrus major*), largemouth bass (*Micropterus salmoides*),

common carp (*Cyprinus cyrpio*), turbot (*Scrophthalmus maximus*) and oyster toadfish (*Opsanus tau*) (Garcia et al., 2012; Park et al., 2012; Xu & Zhang, 2014).

Meanwhile in Malaysia, the bacteria has been successfully isolated from Asian swamp eel, hybrid red tilapia and snakeskin gourami, ornamental fish, giant freshwater prawn, American bullfrog and also African catfish (Najiah, Lee & Lee, 2006; Lee & Najiah, 2008; Najiah, Lee, Faizah & Wendy, 2008; Najiah, Lee, Wendy & Nadirah, 2009; Lee, Najiah, Wendy, Zahrol & Nadirah, 2009a; Lee, Najiah, Wendy, Nadirah & Faizah, 2009b).

2.2.3 Diagnostic Method of Edwardsiellosis Infection

Previously, diagnosis of this highly virulent fish pathogen was done through culture media obtained from the internal organ of diseased fish such as spleen, kidney and intestinal and also gross morphological examination. But since their pathological symptoms are similar to other bacterial infections, further molecular method is required to diagnose this bacterium. There are numerous technique or method for *E.tarda* detection either through traditional bacterial identification such as biochemical test or advanced PCR such as real-time PCR. Serological techniques such as slide agglutination tests and fluorescent antibody technique can also be applied (Park et al., 2012).

Polymerase chain reaction has been the alternative methods used for decades to synthesize million copies of specific DNA sequence. Polymerase chain reaction is a crucial tool in molecular technology to identify bacterial, viral, parasitic and fungal

agents for further pathogenesis characterization, disease detection and laboratory research. Application of PCR has minimized the major weakness in conventional culture technique in which PCR is less time consuming as it does not required the microorganism to be culture and way more effective as it provides high sensitivity, specificity and speed. Nowadays, PCR has gone through modification and development to improve performance, sensitivity and specificity thus produce various advanced method such as real time PCR, semi quantitative PCR and multiplex PCR (Hernandez-Rodriguez & Gomez, 2012).

Application of PCR techniques has its own pros. A fragment of nucleic acid can be simply detected by PCR and allow the sequence to be amplified. Next, PCR are able to produce billions copies of specific DNA sequence for further used for instance sequencing, cloning and other analysis (Garibyan & Avashia, 2013).

Even though PCR has its own advantages but there are also few disadvantages. First of all, due to its high sensitivity techniques, even the slightest amount of any contamination by residual DNA can cause inaccurate results (Smith & Osborn, 2009). On top of that, PCR required some sequence data to create primers that later will be used to detect the absence or presence of only known pathogens or genes (Garibyan & Avashia, 2013). Besides, at a very low rate, incorrect nucleotides can be added to the PCR sequence by DNA polymerase (Garibyan & Avashia, 2013).

The etiological agent of edwardsiellosis through molecular identification has been done by the combination of universal PCR with DNA sequencing. The PCR techniques can be performed using several different primers for instance *E.tarda* universal primer 27F (5’ AGRGTTTGATCMTGGCTCAG 3), 1492R (5’- GGYTACCTT

GTTACGACTT-3') primer targeted the hemolysin gene, hem F (5'-CCTTATAAATTACTCGCT-3'), hem R (5'-TTTGTGGAGTAACAGTTT-3') and also primer for RAPD PCR, Universal M13 (5'-TTATGTAAAACGACGGCCAGT-3'), Wild-type Phage M13 (5'- GAGGGTGGCGGTTCT-3') (Joh et al., 2011; Ibrahem, Shaheed, Yazeed & Korani, 2011; Nadirah, Najiah & Teng, 2012).

Polymerase chain reaction techniques was used in previous research on detection of *E.tarda* including in Asian seabass eel, zebrafish, African catfish and Nile tilapia, Korean catfish, turbot and also Asian clam (Pressley, Phelan, Witten, Mellon & Kim, 2005; Castro, Toranzo, Barja, Nunez & Magarinos, 2006; Yu et al., 2009; Joh et al., 2011; Ibrahem et al., 2011; Nadirah et al., 2012; Lee, Wee, Che Manan, Amin & Hajisamae, 2013).

2.2.4 Signs of Edwardsiellosis Infection

Generally, clinical signs of *E.tarda* infection vary depending on the fish species. Typical clinical sign that can be observed in fish species are popping eye (exophthalmia), degeneration of skin colour, hemorrhages, excessive mucous on skin body, granulomas present in the liver as well as congested internal organ (Xu & Zhang, 2014).

While more specific sign can be observed depends on fish species for example, in infected catfish, they exhibit cutaneous lesion and as the disease develop, abscesses can be seen in the muscle of tail and body. Later, this abscesses become enlarged and filled with hollow gases. On the other side, Nile tilapia display signs such as exophthalmia (pop-eye), lethargy, loss of skin pigment, abdominal swollen filled with ascetic fluid and protruded anus. Internally, both species showed enlargement of

internal organ such as kidney, liver and spleen along with white nodules may be observed on the swollen organ (Ibrahem et al., 2011).

Scientist also performed experimental infection in fish species to study the mortality rate caused by *E.tarda*. During the experimental infection in Japanese flounder fingerling, the scientist immersed the fish in 1.3 x 10^6 bacterial cells/mL of bacterial solution for about 10 minutes. The final result showed a very high mortality rate about 100% within 30 days of post-infection. While *E.tarda* infection occur in a population of young hybrid tilapia reported in Columbia showed mortality rate ranged from 10% up to 90% (Garcia et al., 2012).

2.2.5 Prevention of Edwardsiellosis Infection

The use of antibiotic in preventing edwardsiellosis raise a public concern as administration of antibiotic excessively may develop resistance towards the pathogen. Hence, the antibiotic no longer effective to eliminate the pathogen and cause the pathogen becoming more difficult to defeat. Other than that, the used of antibiotic also create residues that harm the environment and consumer health. However, the used of antibiotic towards *E.tarda* is ineffective since it is an intracellular bacteria and sooner or later, the fish will die (Xu & Zhang, 2014).

Vaccine can be described as a preparation of antigenic substance used to increase immunity against several disease or a specific disease (Park et al., 2012). Recently, there are many research have been done especially in China to produce a competent vaccine in preventing edwardsiellosis infection (Xu & Zhang, 2014). Vaccine

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can be classified into several type which are live attenuated vaccine, killed vaccine, toxoid vaccine and recombinant vaccine. Vary route for administration of vaccine has been applied during the vaccine trials such as intraperitoneal injection, intramuscular injection and oral administrations.

Furthermore, scientist has developed a new method in producing effective vaccine against *E.tarda* by using varied antigenic substances. The trials result showed high relative percent survival (RPS) about 100% survival when challenge with *E.tarda* strains (Park et al., 2012). The used of vaccine together with adjuvant substance is a great alternative to prevent *E.tarda* infection compared to antibiotic.

Besides, it is crucial to maintain an optimum environment in the aquatic population to avoid any stressor factor to the aquatic organisms. Next, it is important for the farmers to purchase broodstock from well-known supplier that provide as much information on the broodstock and have good record keeping. Introduction of infected fish in the population may lead to disease outbreak hence it is too late to prevent the spreading of disease.

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CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Chemical and Reagent

In this study, the chemical and reagent used were PCR reagents (MgCl2, dNTPs 10mm, TAQ DNA Polymerase, 10X TAQ buffer, forward and reverse primer, DEPC water), ethanol 70%, absolute ethanol, 6X Loading dye, agarose powder, set electrophoresis, GelRed Nucleic Acid gel stain, 1X TBE buffer, VC 100bp DNA ladder.

3.1.2 Equipment and Apparatus

The equipment and apparatus used in this study were centrifuge, PCR thermal cycler, thermomixer, UV transilluminator, autoclaves, freezer, vortex, Nanodrop Spectrophotometer, analytical balance, dissecting set, 100 ml beaker, 200 ml beaker, conical flask, gloves, measuring cylinder, chopping board, micropipettes, pipet tips, 0.2 ml PCR microtubes with attached cap, 1.5 ml tubes, GF-1 Tissue DNA Extraction kit.

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3.2 Methodology

3.2.1 Fish Sample

Fish sample were randomly collected from fish pond in UMK Jeli twice a month for a duration of two months sampling period. Approximately, ten hybrid red tilapia weighed range between 141.66 ± 4.02 g and 150.73 ± 3.71 g was collected and transferred to the Aquaculture Laboratory of University Malaysia Kelantan for further isolation and detection of *E.tarda.*

Any abnormalities that can be observed from the fish sampled were recorded during the necropsy. Screening of *E.tarda* was done using kidney tissue of hybrid red tilapia.

3.2.2 DNA Extraction

The DNA was extracted using GF-1 Tissue DNA Extraction Kit prior to manufacturer's protocol (Vivantis Technologies). Briefly, 20mg of tissues sample from kidney of the hybrid red tilapia was cut into smaller pieces. Then, 250 µl of Buffer TL was added along with 20 µl of Proteinase K to the sample followed by mixing by vortexing. Next, 12 µl of Lysis Enhancer was added followed by immediate mixing. The mixture was incubated at 65°C for 1 hour in thermomixer.

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After the incubation, 560 µl of Buffer TB was added followed by mixed through vortexing until homogenous solution obtained. Then, the solution was incubated for 10 minutes at 65°C. After that, 200 µl of absolute ethanol was added and mixed immediately by vortexing. Approximately 650 µl of sample was transferred into the column and centrifuged at 5000 x g for 1 minutes and discarded the flow through. The column was washed with 650 µl of Wash buffer and centrifuged at 5000 x g for 1 minutes and discarded the flow through. The washing step was repeated once again. The column was centrifuged at 10000 x g for 1 minutes to remove all the traces of ethanol.

The column was placed in the clean micro centrifuged tube. Next, 200 µl of Elution buffer was added directly onto the column membrane and stand at room temperature for 2 minutes. Finally, the column was centrifuged at 5000 x g for 1 minutes to elute the DNA and stored the DNA at -20°C.

3.2.3 Primer Synthesis and Polymerase Chain Reaction (PCR)

A region of gyrB gene was amplified using *E.tarda* primers specific for target genes obtained from Park et al. (2014), gyrBF1 (5'- GCATGGAGACCTTCAGCAAT-3'), gyrBR1 (5'- GCGGAGATTTTGCTCTTCTT-3') with expected product sizes, 415bp.

The temperature optimization protocol used was denaturation at 94 °C for 4 minutes, 35 cycles 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 minutes

and final extension at 72 °C for 7 minutes. On top of that, *E.tarda* ATCC isolate was used as positive control in the optimisation protocol.

Polymerase chain reaction amplification was performed in a final volume of 25 µL containing approximately 200 mg of genomic DNA, 21 µL of DEPC water, 1 µL of 10X Taq Buffer , 1 µL of $MgCl_2$, 0.25 µL of each primer, 0.25 µL of dNTPs, and 0.25 µL of Taq DNA polymerase. The PCR mixture was amplified in thermal cycler using optimised PCR protocol, 35 cycles of initial denaturation at 94°C for 4 minutes, denaturation at 94°C for 30 seconds, annealing at 52.8 for 30 seconds, extension at 72°C for 1 minutes, and final extension at 72°C for 7 minutes. The PCR products was determined by 1.5% agarose gel electrophoresis in 1X TBE Running buffer, stained with 0.5µL of GelRed staining and visualized under UV transilluminator.

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CHAPTER 4

RESULT

4.1 Gross Observation of Sampled Fish

Approximately, ten hybrid red tilapia weighed range from 141.66 ± 4.02 g and 150.73 ± 3.71 g were randomly collected from inland farm in the UMK. Necropsy was done at Aquaculture Laboratory of UMK and any abnormalities on external anatomy of hybrid red tilapia was recorded. Each sample was coded based on sampling week such as 1TAK, 2TAK, 3TAK and 4TAK.

Based on the gross observation of the sampled fish, there was no clinical sign that related to edwardsiellosis disease appeared (Figure 4.1). But during the necropsy, congested kidney can be observed from some of the sampled fish from different sampling week (Figure 4.2).

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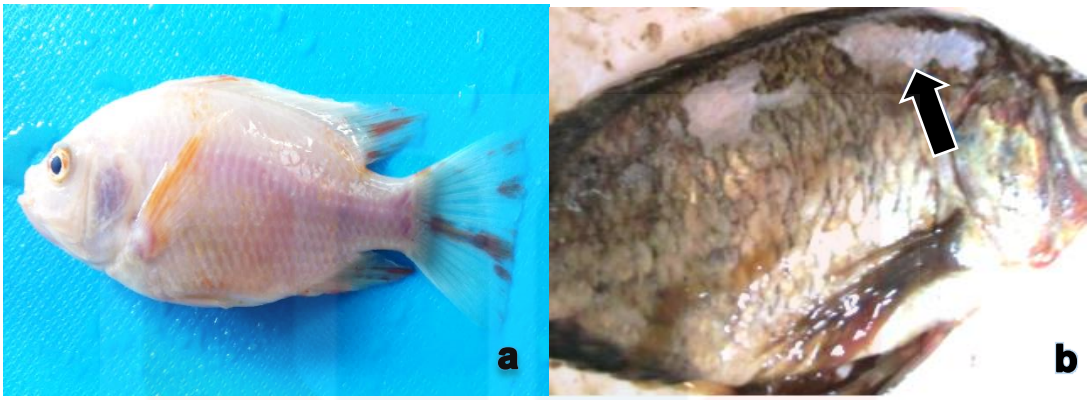

Figure 4.1: (a) Normal external anatomy of hybrid red tilapia and (b) external anatomy of infected Nile tilapia showing cutaneous lesion (arrow) (Moustafa, Omar & Abdo, 2016).

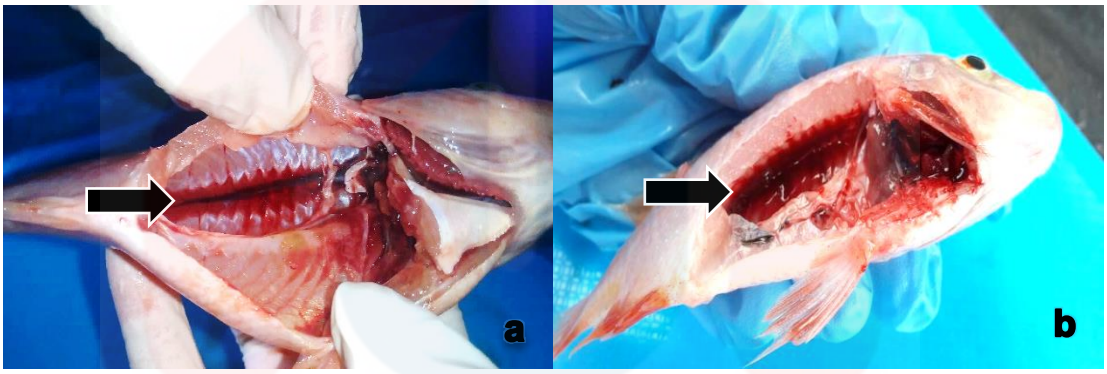

Figure 4.2: Internal anatomy observation: (a) normal kidney in hybrid red tilapia (arrow) and (b) congested kidney in hybrid red tilapia (arrow).

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Table 4.1: Summary of body weight with standard error and internal organ observed from fish sampling.

Sampling ID	Average body weight ± standard error (g)	Internal organ observation
1TAK	149.951 ± 4.454062	3/10 congested kidney
2TAK	150.73 ± 3.711174	2/10 congested kidney
3TAK	141.937 ± 4.05916	1/10 congested kidney
4TAK	141.661 ± 4.019797	1/10 congested kidney

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4.2 DNA Extraction

The DNA was extracted using GF-1 Tissue DNA Extraction Kit and the purity of DNA was checked using NanoDrop Spectrophotometer. There were two ratio used to measure the purity of DNA which are A260/A280 and A260/A230. For the A260/A280 ratio, it was used to assess the protein contamination of the DNA and if the value has greater than or equal value to 1.8 are accepted as pure DNA. Whereas the ratio for A260/A230 was used as a secondary measure and commonly in the range of 1.8 to 2.2.

Based on the result obtained for A260/A280 ratio, the total average of DNA purity from sample Week 1 until sample Week 4 was 1.87. Whereas for A260/A230 ratio, the total average of DNA purity from sample Week 1 until Week 4 fell on 3.03. Overall, the DNA extracted has high value in the A260/A230 ratio indicated presence of large amount contamination while the average for ratio A260/A280 was in general acceptable range.

Next, the extracted DNA from each week of sampling were proceed with DNA quality assessment by gel electrophoresis.

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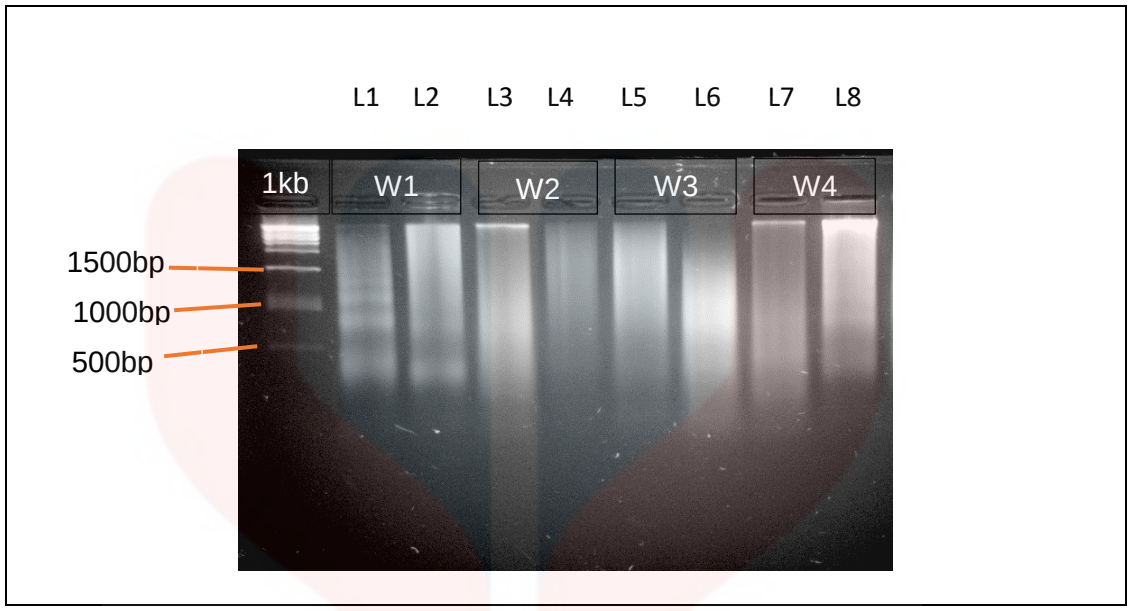

Figure 4.3: Agarose gel electrophoresis showing the purity of DNA sampled using 1kb ladder as DNA marker. Lane 1 = 1TAK1; L2 = 1TAK2; L3 = 2TAK1; L4 = 2TAK2; L5 = 3TAK1; L6 = 3TAK2; L7 = 4TAK1; L8 = 4TAK2.

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4.3 Detection of *E.tarda* by Polymerase Chain Reaction (PCR)

4.3.1 PCR Optimisation

In the present study, the temperature optimisation was mainly focused. The temperature optimisation protocol used was denaturation at 94 °C for 4 minutes, 35 cycles 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 minutes and final extension at 72 °C for 7 minutes. On top of that, *E.tarda* ATCC isolate was used as positive control in the optimisation protocol.

From the result obtained, single band only appeared at temperature ranged from 50.0°C to 55.4°C while no band can be observed for the rest of the temperature (Figure 4.4). At 50.0°C, the single band came into view as the thickest band meanwhile at 55.4°C the single band appeared to be the faintest band.

In order to choose the appropriate annealing temperature to be used in the rest of the PCR protocol, the brightest, well defined band without any nonspecific bands and smear was chose which appeared at temperature 52.8°C in lane five. Hence, the optimised PCR protocol used for screening of *E.tarda* was 94 °C for 4 minutes, 35 cycles denaturation at 94 °C for 30 seconds, annealing temperature at 52.8°C for 30 seconds, extension at 72 °C for 1 minutes and final extension at 72 °C for 7 minutes.

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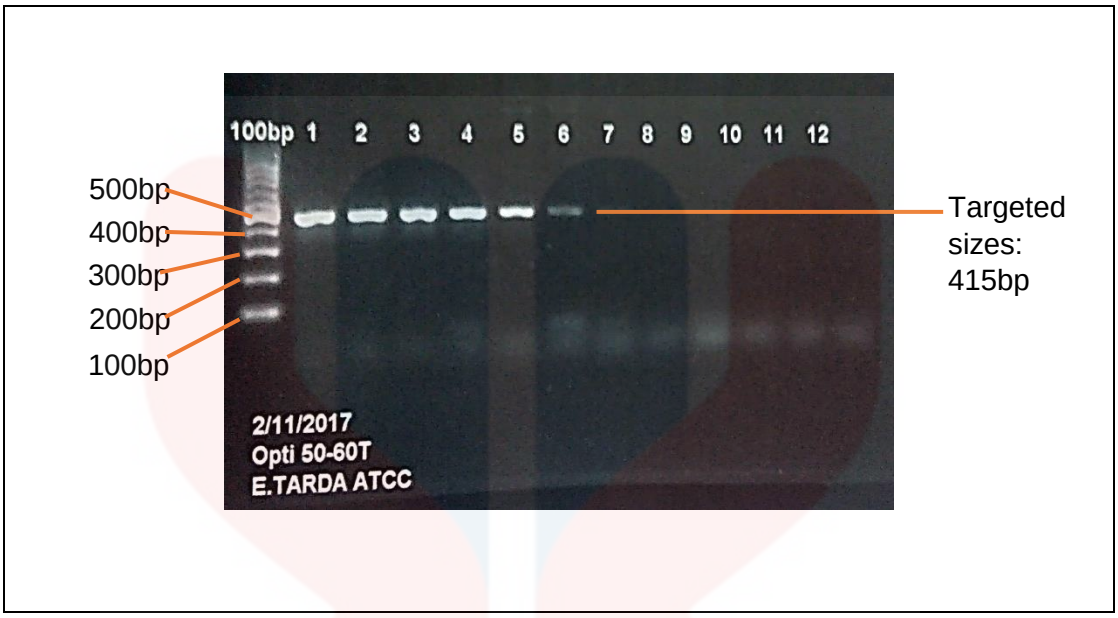

Figure 4.4: Agarose gel electrophoresis showing the optimisation PCR products reaction amplified using primer sets: gyrBF1 and gyrBR1 with expected product of 415bp. 100 bp ladder was used as DNA marker. Lane 1 = 50.0°C ; L2 = 50.2°C ; L3 = 50.8°C ; L4 = 51.7°C ; L5 = 52.8°C ; L6 = 54.1°C ; L7 = 55.4°C ; L8 = 56.7°C ; L9 = 57.9°C ; L10 = 58.8°C ; L11 = 59.5°C ; L12 = 59.9°C.

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4.3.2 Molecular Screening of *E.tarda* Using Optimised Protocol

The present study proceed screening of *E.tarda* using optimised protocol obtained from previous subtopic. The optimised protocol used was 94 °C for 4 minutes, 35 cycles denaturation at 94 °C for 30 seconds, annealing temperature at 52.8°C for 30 seconds, elongation at 72 °C for 1 minutes and final elongation at 72 °C for 7 minutes.

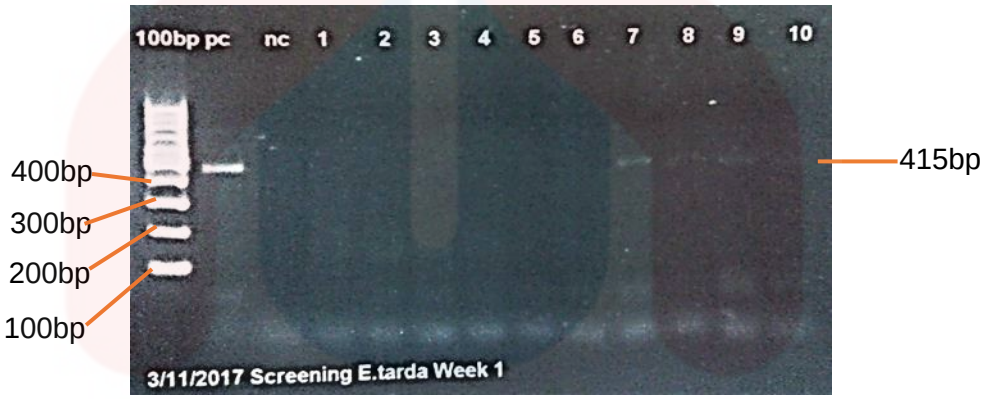

Figure 4.5: Agarose gel electrophoresis showing the product of the polymerase chain reaction of sample from week one amplified using primer sets: gyrBF1 and gyrBR1 with expected product size of 415bp and show that the gyrB1 gene of *E. tarda* was amplified in all isolates used in this study. 100bp ladder = DNA Marker, pc = positive control, nc = negative control; Lane 1 = sample 1; L2 = sample 2; L3 = sample 3; L4 = sample 4; L5 = sample 5; L6 = sample 6; L7 = sample 7; L8 = sample 8; L9 = sample 9; L10 = sample 10.

At the end of PCR screening, the result of *E.tarda* prevalence was tabulated as shown below (Table 4.2). Four out of ten fish sample during Week 1 were detected positive with the presence of *E.tarda*. Whereas sample for Week 2 until Week 4, showed negative PCR result.

Table 4.2: Prevalence of *E.tarda* throughout the sampling period.

Sampling ID	PCR - Positive *E.tarda*	Prevalence (%)
1TAK	4/10	40
2TAK	0/10	0
3TAK	0/10	0
4TAK	0/10	0

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CHAPTER 5

DISCUSSION

Edwardsiellosis caused by one of the most important fish pathogen, *E.tarda* can be characterised by numerous clinical signs depending on the fish species. The most common clinical signs that can be observed were exophthalmia, congested internal organ such as kidney and spleen, ascites, haemorrhages and excessive mucous on the skin surface (Xu & Zhang, 2014).

Based on the gross observation done in this study, the external anatomy of the sampled fish does not shows any clinical sign related to edwardsiellosis disease. However, during the necropsy, congested kidney were observed in some of the sampled fish.

For further diagnosis, DNA of each fish sampled kidney was extracted using GF-1 Tissue DNA Extraction Kit according to the manufacturer's protocol. The extracted DNA then was checked for its purity, quality and quantity via NanoDrop Spectrophotometer. From the result obtained, DNA extracted has low quality as A260/A230 total average was out of general range. For A260/A280 ratio that showed lower value from 1.8, it indicate contamination of residual phenol or any other reagent that were used during the DNA extraction procedures. Whereas for the ratio A260/A230, value that showed higher or lower than the general range, it indicated that there was a presence of unwanted organic contaminants.

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Meanwhile, extracted DNA that was assess for the quality through gel electrophoresis (Figure 4.3) showed the smearing effect that caused by contamination such as presence of alcohol due to incomplete column dried during the column washing. Another reason may due to the presence of RNA because this study does not performed the removal of RNA during the DNA extraction procedure.

In order to obtain desired yield of PCR product, it is essential to make sure the reaction mixtures has appropriate denaturation, annealing and extension temperatures (Kolmodin et al., 2002). Hence, temperature optimisation was done in this study to acquire the best annealing temperature to be used in the PCR screening protocol. If the reaction mixtures fail to amplify under optimum conditions, eventually it will lead to the production of unnecessary products and false positive results (Roux, 2009). A successful PCR amplification is when the single band with expected product size without any smear and multiple band produced.

The PCR protocol used in this study was originally obtained from Park et al. (2014). After the PCR optimisation performed, a well-defined line of band and the brightest band form in the lane five was chosen. The lane five represents the best annealing temperature which was 52.8 °C. The optimised protocol used for the rest of PCR cycles were initial denaturation at 94 °C for 4 minutes, 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 52.8 °C for 30 seconds and extension at 72 °C for 1 minutes followed by final extension at 72 °C for 7 minutes.

Based on the Table 4.1, it found that four out of ten fish sampled were affected with *E.tarda* contributed to the 40% of *E.tarda* prevalence in Week 1. Sampled that affected with *E.tarda* were 1TAK7, 1TAK8, 1TAK9 and 1TAK10. However, only

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sampled number 1TAK9 showed kidney congestion while sampled 1TAK7, 1TAK8 and 1TAK10 does not showed any signed related to *E.tarda* infection. This showed that *E.tarda* presence in the fish but in an amount that does not cause any symptoms to visible.

Furthermore, none of the fish sampled from Week 2 until Week 4 were infected with *E.tarda*. Hence, there was no prevalence of *E.tarda* recorded for the next three weeks. Nevertheless, there were fish sampled that showed congestion of kidney but negative for PCR screening. The congestion of kidney may be caused by other pathogen since it was one of the clinical sign for bacterial infection. Enlargement of kidney can be observed in bacterial infection for instance *Aeromonas hydrophila* and *Streptococcus agalactiae* (Zamri-Saad et al., 2010; Yardimci & Aydin, 2011).

Main factors that can contributes to the onset of the *E.tarda* in a fish population are rise in water temperature, poor water quality, organic content in the water due to increasing of feeding rate, excess feed and overcrowding fish as well as nutritional status of fish. Sometimes, the presence of amphibian in large number in pond can also affect the occurrence of the *E.tarda*. Overcrowding and presence of amphibians increase the microbial load in the water while poor water quality such as salinity, pH and fluctuation in dissolved oxygen also increase the susceptibility of fish towards *E.tarda (*Moustafa et al., 2016).

A sustainable aquaculture production can only take place when the fish is in good health condition and free from disease. There was no history of disease outbreak recorded in the farm. The farmer practiced a good farm management to avoid disease outbreak from occur and minimized as much as possible stressor factor to the fish.

However, during the first week of sampling, the weather was very hot and caused the water temperature to rise. This might be the reason for the presence of *E.tarda* in sample of the first sampling. For the subsequence three week, the weather started to rain almost every day and the water temperature dropped from usual.

Even though there was low prevalence of *E.tarda* detected in the sample, it still can lead to outbreak of disease in the farm if not properly monitored. Since *E.tarda* can be found in humans and other animal species, it shows that *E.tarda* is a potential zoonotic pathogen. Especially if the infected fish or the carrier fish in consumed by humans, it will lead to diarrhea illness and vomiting. Even worse incidence can happen if the consumer is pregnant. Neonatal infection may occur from the consumption that eventually will lead to the death of the infant. Direct contact with the bacteria also bring harm to the human since it cause extraintestinal disease if the bacteria successfully enter the host through body wound.

Thus, development of prevention method against this pathogen deserved a serious attention among the farmers. Appropriate control is needed to eliminate this pathogen to ensure the public health of consumers including the future of commercial aquaculture industry by make sure the *E.tarda* infection is under controlled.

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CHAPTER 6

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

This study was conducted to determine the prevalence of *E.tarda* in hybrid red tilapia from inland farm in Jeli, Kelantan. Edwardsiellosis disease has significant impacts toward aquaculture industry causing high percentage of mortalities between 10% and 90% mortality. Not only the farmers suffer a significant loss due to this disease, it also affected the health of consumer that consumed the infected fish. Previous studies have reported several cases of gastrointestinal infection caused by this bacterium.

This study had successfully isolated *E.tarda* from the kidney of fish sampled hence detected 40% of *E.tarda* prevalence from the sampling site throughout the sampling period. However, the prevalence only represent the fish sampled from Week 1 and no prevalence were obtained for the rest of the sampling period.

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6.2 Recommendation

The finding from this study can be further confirm with traditional technique in bacterial detection such as biochemical test. Since molecular identification technique such as conventional PCR may sometimes obtained a false positive result, it is better to combine the molecular technique with traditional method to double confirm the presence of *E.tarda*. Biochemical test can be done at the same time during screening of *E.tarda* using PCR. Hence, biochemical characteristic of *E.tarda* isolated can be determined.

In addition, the DNA must be handle correctly to avoid any unwanted contamination. Especially during the extraction procedure, make sure the column drying was completely dry as presence of excess alcohol will cause smearing effect to be visible during the assessment of DNA purity via gel electrophoresis. This will make the DNA quality to be low and may not suitable to be used for the PCR amplification. It is also recommended to extract the DNA from more internal organ such as liver and intestine so that the distribution of this bacteria inside the fish can be observed. Besides, optimisation of each reagents can also be performed along with the temperature optimisation.

Next, the sampling period can be prolonged so that the distribution pattern of *E.tarda* can be observed. The sampling period for this study was only for two months and the distribution of *E.tarda* cannot be observed whether it presence the most during hot and drought season or during rainy season. Prolonged the sampling period can add more validity to this study as more information and prevalence can be obtained.

On top of that, it is recommended to increase the sampling area outside from Jeli and target more commercial fish farm in Kelantan district. The study can also be improved by comparing the culture system used by the farmers with the prevalence of *E.tarda*. Hence, better understanding on this bacterium can be obtained.

Moreover, the presence of *E.tarda* in the fish pond warrants further considerable measure to be taken by the farmers to avoid spreading of this bacterium to nearby farm. Hence, regular assessment regarding to the farm management, water quality parameter and other component that trigger stressor factor to the fish population must be done. Component that will increase the prevalence and severity of *E.tarda* infection must be considered in designing the prevention method and disease management.

Since the current knowledge on this bacterium in Kelantan is relatively low, further study must be done in the future for the benefits of public health consumer as well as the aquaculture activity so that outbreak of edwardsiellosis can be controlled and prevented.

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APPENDIX

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

Farm	Code	Fish Weight (g)	Observation		PCR
			External	Internal	
A	1TAK1	152.50	Normal skin pigmentation, eyes and fin.	Normal	Negative
A	1TAK2	125.30	Normal skin pigmentation, eyes and fin.	Normal	Negative
7A	1TAK3	137.50	Normal skin pigmentation, eyes and fin.	Kidney enlarged	Negative
A	1TAK4	145.00	Normal skin pigmentation, eyes and fin.	Kidney enlarged	Negative
A	1TAK5	150.70	Normal skin pigmentation, eyes and fin.	Normal	Negative
A	1TAK6	173.30	Normal skin pigmentation, eyes and fin.	Normal	Negative
A	1TAK7	145.70	Normal skin pigmentation, eyes and fin.	Normal	Positive
A	1TAK8	155.43	Normal skin pigmentation, eyes and fin.	Normal	Positive
A	1TAK9	149.33	Normal skin pigmentation, eyes and fin.	Kidney enlarged	Positive
A	1TAK10	164.75	Normal skin pigmentation, eyes and fin.	Normal	Positive

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

Farm	Code	Fish Weight (g)	Observation		PCR
			External	Internal	
A	2TAK1	142.37	Normal skin pigmentation, eyes and fin.	Normal	Negative
A	2TAK2	150.00	Normal skin pigmentation, eyes and fin.	Normal	Negative
A	2TAK3	169.30	Normal skin pigmentation, eyes and fin.	Normal	Negative
A	2TAK4	136.75	Normal skin pigmentation, eyes and fin.	Normal	Negative
A	2TAK5	144.00	Normal skin pigmentation, eyes and fin.	Enlargement of kidney	Negative
A	2TAK6	165.43	Normal skin pigmentation, eyes and fin.	Normal	Negative
A	2TAK7	137.90	Normal skin pigmentation, eyes and fin.	Normal	Negative
A	2TAK8	159.60	Normal skin pigmentation, eyes and fin.	Normal	Negative
A	2TAK9	151.20	Normal skin pigmentation, eyes and fin.	Normal	Negative
A	2TAK10	150.75	Normal skin pigmentation, eyes and fin.	Enlargement of kidney	Negative

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

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

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

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

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Table A.5: DNA Extraction for sample Week 1 through Nano drop.

Code	Organ	DNA/ng	260/280	260/230
1TAK1	Kidney	62.9	1.87	3.15
1TAK2	Kidney	24.1	1.70	3.92
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.6: DNA Extraction for sample Week 2 through Nano drop.

Code	Organ	DNA/ng	260/280	260/230
2TAK1	Kidney	30.3	1.94	2.80
2TAK2	Kidney	34.4	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	30.5	1.86	2.78
2TAK10	Kidney	11.6	1.79	2.53

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Table A.7: DNA Extraction for sample Week 3 through Nano drop.

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.8: DNA Extraction for sample Week 4 through Nano drop.

Code	Organ	DNA/ng	260/280	260/230
4TAK1	Kidney	13.2	1.85	2.36
4TAK2	Kidney	30.3	1.98	2.49
4TAK3	Kidney	20.3	1.64	2.37
4TAK4	Kidney	35.9	1.85	3.19
4TAK5	Kidney	57.4	1.74	2.63
4TAK6	Kidney	16.7	1.82	2.54
4TAK7	Kidney	40.0	1.78	3.86
4TAK8	Kidney	12.8	1.73	2.43
4TAK9	Kidney	17.6	1.80	3.42
4TAK10	Kidney	26.6	1.79	1.30

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