UNIVERSITI PUTRA MALAYSIA

ISOLATION, CHARACTERIZATION AND PATHOGENICITY OF EPIZOOTIC ULCERATIVE SYNDROME-RELATED Aphanomyces TOWARD AN IMPROVED DIAGNOSTIC TECHNIQUE

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SEYEDEH FATEMEH AFZALI

Thesis Submitted to the School of Graduate Study, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Doctor of Philosophy

August 2014

DEDICATION

This dissertation is lovingly dedicated to my kind family.

A special feeling of gratitude to my great parents who inspired my life through their gritty strength, enduring faith, and boundless love for family. My nice sisters and brother have never left my side and have supported me throughout the process. I also dedicate this work and give special thanks to my best friend “Hasti” for being there for me throughout the entire doctorate program.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

ISOLATION, CHARACTERIZATION AND PATHOGENICITY OF EPIZOOTIC ULCERATIVE SYNDROME-RELATED *Aphanomyces* TOWARD AN IMPROVED DIAGNOSTIC TECHNIQUE

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Epizootic ulcerative syndrome (EUS) is a seasonal and severely damaging disease in wild and farmed freshwater and estuarine fishes. The disease has been spread through countries of the Asia-Pacific region with dire consequences to the fish resources and livelihood of fishermen. It has been a major concern almost all over the world since 1972. Epizootic ulcerative syndrome is a disease which manifested with severe skin and muscle ulceration and caused heavy mortalities in freshwater fishes. The aquatic fungus, *Aphanomyces invadans*, which belongs to the family *Saprolegniacea*, has been identified as the causative agent of EUS. Up to date no effective prophylactic measures and no protective vaccines are available against this disease. If scientific development could not solve this microbiological problem, it is likely to impact a noticeable negative income in the future especially for fish farmers who rely on wild-caught fish for income. Thus this study aimed to (i) isolate and identify *Aphanomyces* spp. from Malaysian water bodies and fish farms, (ii) determine the pathogenicity of *A. invadans* on the Malaysian local fish, and (iii) improve a molecular technique (PCR) for a rapid and reliable detection of EUS infection.

Four hundred sixty one water and 235 fish were sampled from different water bodies and fish farms in Selangor state of Malaysia from February 2011 until February 2013. Oomycete fungi were isolated by applying bait methods using hempseed and corn, and identified according to their hyphae, sporangium and oogonium morphological characteristics.

Through experimentally infection studies, Snakehead fish (*Channa striata*) (positive control), Moonlight gourami (*Trichopodus microlepis*), Snakeskin gourami (*Trichopodus pectoralis*), Koi carp (*Cyprinus carpio carpio*), Broadhead catfish (*Clarias macrocephalus*), Goldfish (*Carasius auratus auratus*), Climbing perch (*Anabas testudineus*) and Tilapia (*Oreochromis niloticus*) (negative control) were challenged by intramuscular injection and cohabitation using zoospores of a reference *A. invadans* NJM9701 (isolated from naturally infected Ayu by Dr. Hatai in Japan, 1997). *Aphanomyces invadans* was able to be re-isolated from experimentally infected Moonlight gourami and Koch's postulates were fulfilled to confirm the exact source of infection in this study. *Aphanomyces invadans* DNA were extracted from experimentally infected fish skin and muscle at different days of post

inoculation and were detected by the PCR method by using the primer set 1APM 1 F, 1APM 6R which were commercially available in the market.

From 73 water samples which were positive for fungi, 31 isolates were identified as *Saprolegnia* spp., 27 isolates as *Achlya* spp., 12 isolates as *Aphanomyces* spp., and three isolates as *Allomyces* spp. Among of 235 naturally infected fish, 62 samples were positive for fungi infection which identified as *Saprolegnia* (34 samples) and *Achlya* (28 samples). Snakeheads experimentally infected with local isolates of *Aphanomyces* did not show any EUS typical clinical signs and no mortalities were observed in any group during observation period, which indicated that the local isolates of *Aphanomyces* spp., were of saprophytic strains. Snakehead, Gouramy, Koi carp, Broadhead catfish, Goldfish and Climbing perch injected with zoospores from reference strain developed lesions that were grossly and histopathologically identical to those observed in naturally infected fish and 100% mortalities were observed. Histopathological studies showed severe cellular inflammatory infiltration, granulomatous formations and presence of invasive fungal hyphae in zoospores injected fish skins and muscles. The DNA extraction protocol used in this study was successful in isolating *A. invadans* genomic DNA from fish muscles and pure cultured fungus, and the improved PCR assay also was able to detect the presence of *A. invadans* DNA in experimentally infected fish skin and muscle from day one post inoculation.

This study was the first research conducted on freshwater aquatic fungi in Malaysia and successfully showed the presence of *Aphanomyces* spp., and other oomycete fungi in Malaysian water bodies. It is found that Malaysian Moonlight gourami, Snakeskin gourami, Koi carp and Broadhead catfish are highly susceptible while Goldfish and Climbing perch are moderately susceptible to infection by *A. invadans* via intramuscular injection. The infection is also capable of being transferred to healthy susceptible fish through the water column. It is concluded that by applying PCR assay *A. invadans* could be detected in clinical samples in very early stages of disease. Because of the presence of *Aphanomyces* spp., and EUS-susceptible fish in freshwater resources of Malaysia, there is potential risk of EUS outbreak in the region which thus must be avoided by good prophylactic measures and rigid farm biosecurity.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMENCILAN, PENCIRIAN DAN PATOGENISITI *Aphanomyces* BERKAITAN SINDROM EPIZOOTIK ULSERATIF, KE ARAH PENAMBAIKAN TEKNIK DIAGNOSTIK

Oleh

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Sindrom epizootik ulseratif (EUS) adalah penyakit bermusim dan membawa kerosakan yang serius dalam ikan air tawar dan muara yang diternak atau liar. Penyakit ini telah merebak ke serata negara di kawasan Asia-Pasifik dengan kesan yang menakutkan kepada sumber perikanan dan kehidupan nelayan. Ianya telah menjadi perhatian utama di seluruh dunia semenjak tahun 1972. Sindrom epizootik ulseratif adalah penyakit yang menunjukkan ulser teruk di kulit dan otot dan menyebabkan kematian yang tinggi dalam ikan air tawar. Fungus *Aphanomyces invadans*, yang tergolong dalam keluarga *Saprolegniacea* telah dikenalpasti sebagai agen penyebab EUS. Setakat ini tidak ada langkah profilaktik yang efektif dan vaksin perlindung terhadap EUS. Jika pembangunan saintifik tidak berjaya menyelesaikan masalah mikrobiologi ini, ianya akan memberi impak negatif yang jelas pada masa hadapan terutama kepada yang bergantung terhadap tangkapan ikan liar sebagai sumber pendapatan. Oleh itu kajian ini bertujuan untuk (i) memencil dan mengenalpasti spesis *Aphanomyces* daripada perairan dan ladang ikan, (ii) menentukan patogenisiti *A. invadans* dalam ikan tempatan Malaysia, dan (iii) peningkatkan teknik molekular (PCR) untuk pengesanan jangkitan EUS secara cepat dan kebolehpercayaan.

Sampel air dan ikan telah diperoleh di perairan dan ladang ikan yang berlainan dari Februari 2011 sehingga Februari 2013 dalam Selangor, Malaysia. Kulat Oomycete dipencilkan melalui kaedah umpan menggunakan biji hemp dan jagung dan ia dikenalpasti mengikut pencirian hifa, sporangium dan oogonium.

Melalui kajian infeksi artifisial, ikan Haruan (*Channa striata*), Gourami bulan (*Trichopodus microlepis*), Gourami kulit ular (*Trichopodus pectoralis*), ikan Koi (*Cyprinus carpio var. carpio*), ikan Keli bunga (*Clarias macrocephalus*), ikan Mas (*Carasius auratus var. auratus*), ikan Puyu (*Anabas testudineus*) dan Tilapia (*Oreochromis niloticus*) telah disuntik dengan zoospora rujukan *A. invadans* (NJM9701) secara intraotot dan jangkitan secara kohabitasi. Untuk memenuhi postulat Koch, *A. invadans* telah berjaya diisolasi semula daripada ikan Gourami bulan yang telah dijangkiti secara artifisial. Asid deoksiribonukleik (DNA) *A. invadans* telah diekstrak daripada tisu ikan tersebut pada peringkat infeksi penyakit yang berbeza dan dikenalpasti melalui kaedah reaksi berantai polimerase (PCR) menggunakan pasangan primer “1APM 1 F, 1APM 6R” yang ada dalam pasaran.

Daripada 73 sampel air positif kulat, 31 isolat telah dikenalpasti sebagai *Saprolegnia* spp., 27 isolat *Achlya* spp., 12 isolat *Aphanomyces* spp., dan tiga isolat *Allomyces* spp. Dalam kalangan 235 ikan yang terinfeksi, 62 sampel adalah positif kulat *Saprolegnia* (34 sampel) dan selebihnya adalah *Achlya* (28 sampel). Ikan haruan yang telah dijangkiti dengan isolat *Aphanomyces* tempatan tidak menunjukkan sebarang tanda klinikal lazim EUS dan tiada sebarang kematian dilihat sepanjang tempoh pemerhatian dalam mana-mana kumpulan ikan. Ini menunjukkan bahawa isolat *Aphanomyces* spp. tempatan adalah strain saprofitik. Ikan Haruan, Gourami, Koi dan Keli bunga, ikan Mas, dan Puyu yang telah disuntik dengan zoospora rujukan dilihat mengalami lesi yang serupa dengan simptom yang ada pada ikan dijangkiti EUS secara semulajadi dan 100% kematian telah dicatatkan. Pemerhatian histopatologi menunjukkan terdapat inflamasi susupan sel yang teruk, pembentukan granuloma dan kehadiran hifa kulat invasif pada kulit dan otot ikan yang disuntik zoospora. Kaedah pengekstrakan DNA yang digunakan telah berjaya memencilkan DNA genomik *A. invadans* dari otot ikan dan kulat yang dikultur, dan asai PCR juga berjaya mengenalpasti kehadiran DNA *A. invadans* daripada tisu ikan yang dijangkiti secara artifisial mulai hari pertama selepas suntikan.

Kajian ini merupakan kajian ulung yang dilakukan terhadap kulat akuatik air tawar di Malaysia dan telah berjaya membuktikan kehadiran *Aphanomyces* spp. dan kulat oomysit yang lain dalam perairan di Malaysia. Selain itu, didapati ikan Gourami bulan, Gourami kulit ular, Koi dan Keli bunga sangat peka terhadap infeksi *A. invadans* secara intarotot manakala ikan Mas dan Puyu adalah sederhana peka. Infeksi ini juga didapati mudah untuk menjangkiti ikan peka yang sihat melalui kolum air. Asai PCR yang dilakukan sangat sensitif terhadap kehadiran *A. invadans* dalam sampel klinikal dan dapat dikenalpasti pada peringkat awal jangkitan lagi. Disebabkan kulat spesis *Aphanomyces* dan ikan peka-EUS dijumpai di dalam sumber air tawar di Malaysia, terdapat kemungkinan berlakunya ledakan penyebaran EUS di kawasan ini. Justeru, langkah profilatik yang baik dan kaedah penternakan yang mementingkan biokeselamatan perlulah diberi perhatian.

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I certify that a Thesis Examination Committee has met on 13 August 2014 to conduct the final examination of Seyedeh Fatemeh Afzali on her thesis entitled "Isolation, Characterization and Pathogenicity of Epizootic Ulcerative Syndrome-Related *Aphanomyces* Toward an Improved Diagnostic Technique" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

AAHRI	Aquatic Animal Health Research Institute, Thailand
ACIAR	Australian Centre for International Agriculture
APW	Autoclaved pond water
*Cs*Casp10	*Channa striata* Caspase 10 (amino acid)
EDTA	Ethilen diamina tetraacetic acid
EFSA	European Food Safety Authority
EUS	Epizootic ulcerative syndrome
FAO	Food and Agriculture Organisation of the United Nations
GPY	Glucose peptone yeast
GP	Glucose peptone
GY	Glucose yeast
H&E	Haematoxylin and Eosin
IFAT	Immunofluorescence antibody technique
MAb	Monoclonal antibody
MG	Mycotic granulomatosis
MGC	Multinucleate giant cell
MW	Molecular weight
OIE	Office Internationale des Epizooties
PAb	Polyclonal antibody
PBS	Phosphate buffered saline
PG-1	Peptone glucose media one
PDA	Potato Dextrose Agar
RAPD	Random amplification of polymorphic DNA
RSD	Red spot disease
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophorsis
TBS	Trizma buffered saline
UM	Ulcerative mycosis
UV	Ultra-violet

CHAPTER 1

INTRODUCTION

Epizootic Ulcerative Syndrome (EUS) is a dangerous fish disease of wide range of fresh and brackish water wild and farmed fish throughout the world. It causes serious economic losses in many countries during the last four decades (Baldock *et al*., 2005). The first EUS onset was reported in Japan in 1971, and later in 24 countries within four continents, viz. Northern America, Southern Africa, Asia and Australia (OIE, 2013; Oidtmann, 2011). *Aphanomyces invadans* is a causative agent of EUS (Saylor, 2010; Baldock *et al*., 2005; Ahmed and Hoque 1999; Lilley *et al*., 1997) by producing a proteolytic enzyme that helps it to penetrate the fish tissue causing shallow to deep ulcers (Chinabut and Roberts, 1999), leading to high mortality in fish population (Kamilya and Baruah, 2013).

The actual amount of economic losses in the aquaculture industry worldwide due to EUS is estimated to be just over USD 9 billion (Harikrishnan *et al.,* 2010) per year, which is about 15% of the value of the world's farmed fish and shellfish production. Furthermore, decreasing fish biomass causing unchangeable damage to the aquatic biodiversity is some indirect impacts of this destructive disease.

Diagnosis of EUS is difficult, as this fungus does not produce sexual structure which is essential for morphological identification. Thus, diagnosis done by observation granulomatous response in histopathology sections and must be confirmed by polymerase chain reaction (PCR) amplification. For rapid detection of uncultivable or fastidious microorganisms and characterization of the pathogen, PCR-based systems which detect the etiologic agents of disease directly from clinical samples, without the need for culture, have been useful (Tang *et al.,* 1997). It is also very specific due to the nature and orientation of the oligo-nucleotide primers that are required to allow amplification to proceed (Shariff *et al.,* 2000). Polymerase chain reaction techniques may solve the problems associated with the identification of pathogenic *A. invadans* which is so difficult and time consuming (Kuan *et al.,* 2013; Phadee *et al.,* 2004).

Epizootic ulcerative syndrome is a worldwide disease and has high mortality in farmed and wild fish. The control of disease in wild fish populations in open water bodies is most likely impossible (Fairweather, 1999), however, it is based on water treatment and management strategies (Lilley *et al*., 1998). On the other hand, there is no effective prophylactic measure for *A. invadans*-infected fish in the wild and in aquaculture ponds. Attempts at using green water, ash, lime, salt (Noga, 2010) and neem (*Azadirachta indica*) seeds or branches for prophylactic treatments of the EUS-infected fish in fish ponds gave variable results (Clifton and Alderman, 2006), and accumulation of these residues cause pollution and made consumers reluctant during the last few years. There is no protective vaccine available (OIE, 2013), however, Snakehead fish that had been immunized with an extract of *A. invadans* elicited humoral immune response (Arockiaraja *et al.,* 2012; Thompson *et al.,* 1997). Since vaccinations are also complicated and expensive method, at present it could not be practical way for prevention EUS (Newman *et al*., 2003).

So, if scientific development could not solve this ecological problem, it is likely to impose a noticeable cost in the future to the next generation especially for farmers who rely on fishing for income and fisher's livelihood and so on people's health. It can be expected to culture EUS resistant fish species in fish farms in the coming future to decrease fish losses arising from EUS outbreak.

Epizootic ulcerative syndrome was reported for the first time in Southern Peninsular Malaysia in 1979 and later, in rice-field fishes in Northern Malaysia and affected some Malaysian important fish like Snakehead, Snakeskin gourami, Catfish and Anabas (Lilley *et al*., 1998), but no scientific work was done on EUS until present. In addition the other reason which led us to conduct research on EUS in Malaysia is that EUS is listed in OIE aquatic animal diseases list (OIE, 2013) and all OIE member countries (including Malaysia) are obliged to conduct research on OIE listed diseases to make an official report for any occurrence of disease. So far, there has been conducted no studies on the aquatic pathogenic oomycetes specially EUS *Aphanomyces* in Malaysia which has high production and international trade of fish in the world (Ng and Tan, 1997). International trade in aquaculture animals still causes spread of major infectious diseases. Further un-restricted trade in aquatic animals without the knowledge of whether the animals from one country to another serve as a vector for a particular disease are already having a major negative impact on aquaculture (Eli, 2008). This research was conducted in three main chapters; the first chapter investigated EUS related *Aphanomyces* infection (ERA) in a selected area of Malaysia (Selangor state) where is economically important in terms of aquaculture and fish industry. The second chapter aimed to fulfill experimental infection studies on Malaysian local fish to investigate the susceptibility of selected fish to EUS. Finally, the third chapter aimed to establish and improve molecular method for detection of EUS in fish.

However, a number of studies have done on EUS in the world to isolate and characterize the etiological agent of EUS, but Malaysian local fish have not previously been experimentally challenged and the potential impact of an introduction of the pathogen into Malaysia on wild and farmed fish populations is unclear. Hence, the general aim of this study is characterization and isolation of *Aphanomyces* spp., and establishing diagnostic technique for detection *A. invadans* to gain insights into the EUS and Malaysian local fish susceptibility to this world wild disease, in order to create a technical pathway for future study on EUS and decrease economical impacts associated with EUS likely onset in Malaysia.

1.1 Objectives

Current study was conducted to:

1. Isolate, identify and characterize *Aphanomyces* spp. from fish and water in Selangor state, Malaysia.
2. Determine pathogenicity of isolated *Aphanomyces* spp. to the most susceptible fish to the EUS (Snakehead, *Channa striata*).
3. Assess the virulence of *A. invadans* strain in the most important local fishes of Malaysia (Snakehead, Moonlight gourami, Snakeskin gourami, Koi Carp, Goldfish, Broadhead catfish, Climbing perch and Tilapia).
4. Establish a PCR method for rapid and reliable diagnosis of *A. invadans*.

1.2 Hypothesis

Aquatic fungi infections are common in Malaysian water bodies, and local Malaysian freshwater fishes are susceptible to the *Aphanomyces invadans* infection.

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