UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERIZATION AND EXPERIMENTAL INFECTION OF INFECTIOUS SPLEEN AND KIDNEY NECROSIS VIRUS FROM ORNAMENTAL FISH IN PENINSULAR MALAYSIA

KUTTICHANTRAN A/L SUBRAMANIAM

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By

KUTTICHANTRAN A/L SUBRAMANIAM

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

July 2014

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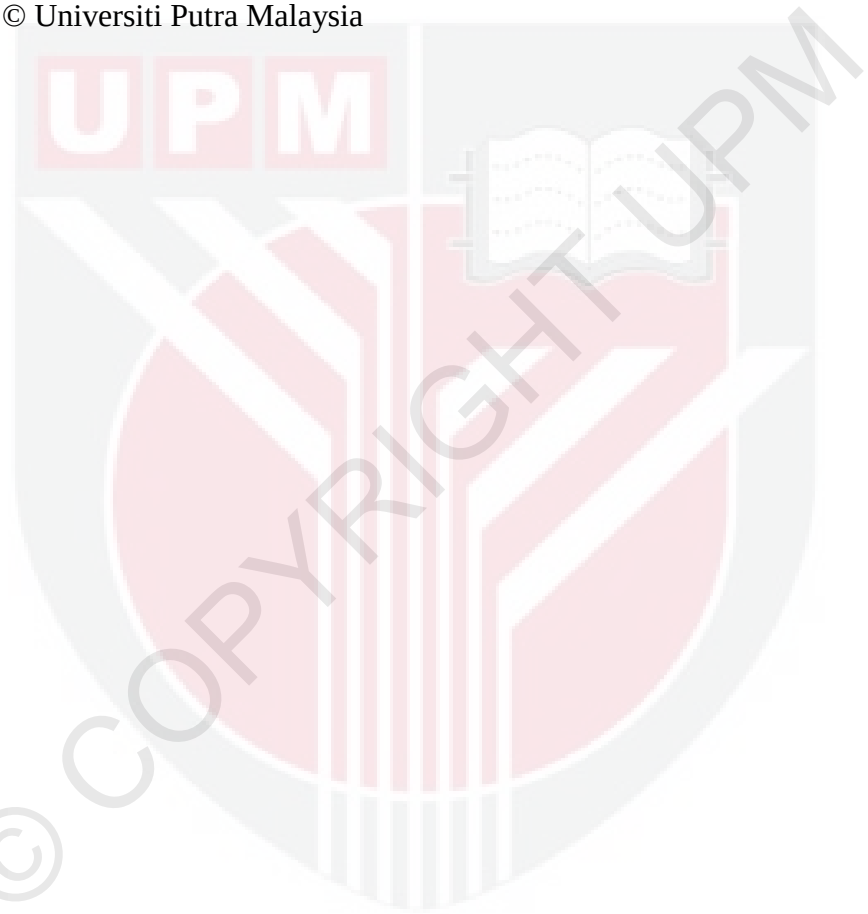

DEDICATION

To my parents, Subramaniam and Susila, for their unending love and support.

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

MOLECULAR CHARACTERIZATION AND EXPERIMENTAL INFECTION OF INFECTIOUS SPLEEN AND KIDNEY NECROSIS VIRUS FROM ORNAMENTAL FISH IN PENINSULAR MALAYSIA

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KUTTICHANTRAN A/L SUBRAMANIAM

July 2014

Chairman: Professor Dato' Mohamed Shariff Bin Mohamed Din, PhD

Faculty of Veterinary Medicine

Infectious spleen and kidney necrosis virus (ISKNV) has been reported in the ornamental fish and this virus belongs to the genus *Megalocytivirus*. Even though this virus have been reported in many countries such as Japan, China, Korea, Taiwan, Thailand and Singapore, the impact and extent of this disease is unknown hitherto at Malaysia. This is due to lack of knowledge on the host range, geographical distribution and the differences between strains if any. Hence to elucidate this gap of knowledge, 'gold standard' OIE reference polymerase chain reaction (PCR) assay was utilized to detect the presence of ISKNV in farmed ornamental fish from Peninsular Malaysia. A total of 210 ornamental fish samples were collected. Of these, ISKNV was detected in 36 ornamental fish samples and they were asymptomatic. Three restriction enzymes analyses showed that the fish were infected by identical strains of same virus species within *Megalocytivirus* genus. Major capsid protein (MCP) gene of 10 ISKNV strains were sequenced and compared with 9 other reference nucleotide sequences acquired from GenBank. Sequence analysis of MCP gene showed that all strains detected in this study were closely related to the reference ISKNV with nucleotide sequence homology ranging from 99.8 % to 100 %. In addition, phylogenetic analysis of MCP gene revealed that the reference ISKNV which was obtained from GenBank and all other strains that were detected in this study were included in genotype 1.

Since all the infected fish appeared healthy, there was a concern over possible transmission of asymptomatic ISKNV infection in freshwater ornamental fish species. To clarify this, an experimental trial was conducted to investigate the possible transmission of ISKNV infection in ram cichlid by cohabitation. The ISKNV is able to transmit from treated fish to cohabited fish within first week of trial and the infected fish were asymptomatic. The presence of ISKNV in the experimentally infected fish was confirmed by PCR assay and histopathology. The ISKNV carrier pose serious risk to the Malaysian aquaculture industry as this virus can spread without any sign of disease. The

inclusion body-bearing cells (IBCs) which are pathognomonic for *Megalocytivirus* infection were present in the liver and spleen. In addition, other histopathological changes such as accumulation of inflammatory cells in splenic pulp and well defined melano-macrophage centers varied from yellow-brown to black deposition of melanin were noted in the spleen.

Visual inspection for clinical signs is not suitable to monitor ISKNV infection as this disease can be asymptomatic in fish. Hence, a highly specific and simple loop-mediated isothermal amplification (LAMP) method was developed in this study for the detection of ISKNV. A set of four primers was designed based on the ISKNV MCP gene sequences. The optimum temperature and time for the LAMP assay were 65 ºC and 60 min, respectively. This assay does not require any sophisticated equipments and allows the investigators to carry out the diagnostic test at farm. Compared to other molecular diagnostic methods such as PCR and qPCR, the reaction time for LAMP assay is shorter and gives instant result without the need of any lengthy post reaction procedures. Accurate identification of the pathogens using highly specific diagnostic tool is paramount to control the spread of infectious diseases. One of the advantages of present LAMP assay was its specificity towards ISKNV. The primers were specific for ISKNV and there was no cross amplification with red sea bream iridovirus (RSIV), white spot syndrome virus (WSSV), *Aeromonas hydrophila* or *Vibrio parahaemolyticus*. The detection limit of LAMP assay was 20 fg. Diluted acridine orange was used to detect the presence of amplified product and this novel step turns the amplified LAMP product into yellow indicating positive reaction and remains orange on negative reaction. In addition, usage of acridine orange in LAMP product gives clear qualitative result which can be visualized without the aid of special lighting or agarose gel electrophoresis.

In summary, the extent of ISKNV infection in farmed ornamental fish which includes information on the host range and geographical distribution in Peninsular Malaysia has been revealed in this study. This baseline information is essential to mitigate the spread of this disease. Present study also confirms the transmission of the asymptomatic ISKNV infection in ram cichlid by cohabitation. There were no previous reports on the transmission of asymptomatic ISKNV by cohabitation. The current LAMP technique for the detection of ISKNV is a simple, specific and inexpensive diagnostic tool under laboratory conditions and also in the field.

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

PENCIRIAN MOLEKUL DAN UJIKAJI JANGKITAN VIRUS INFECTIOUS SPLEEN AND KIDNEY NECROSIS DARIPADA IKAN HIASAN DI SEMENANJUNG MALAYSIA

Oleh

KUTTICHANTRAN A/L SUBRAMANIAM

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Pengerusi: Profesor Dato' Mohamed Shariff Bin Mohamed Din, PhD

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Infectious spleen and kidney necrosis virus (ISKNV) telah dilaporkan di industri ikan hiasan dan virus ini diklasifikasikan dalam genus *Megalocytivirus*. Walaupun virus ini dilaporkan di Jepun, China, Korea, Taiwan, Thailand dan Singapura, kesan dan tahap jangkitan masih tidak ketahui di Malaysia. Ini disebabkan oleh kekurangan pengetahuan perumah, taburan geografi dan perbezaan antara strain jika ada. Jadi dalam kajian ini, ujian PCR 'gold Standard' daripada OIE telah digunakan untuk mengesan ISKNV pada ikan hiasan yang diternak dari Semenanjung Malaysia. Sejumlah 210 sampel ikan hiasan yang diternak di Semenanjung Malaysia telah dikumpul. Daripada itu, ISKNV telah dikesan pada 36 sampel dan ianya asimptomatik. Analisis dengan menggunakan tiga jenis enzim penghad menunjukkan kesemua ikan dijangkiti oleh spesies virus yang terdiri daripada strain yang sama dari genus *Megalocytivirus*. Gen protein kapsid utama (MCP) daripada 10 ISKNV strain telah dijujuk dan telah dibandingkan dengan 9 urutan nukleotida rujukan yang diperolehi daripada GenBank. Analisis urutan gen MCP menunjukkan bahawa kesemua isolat yang dikesan dalam kajian ini mempunyai kadar persamaan urutan nukleotida antara 99.8% hingga 100% apabila dibandingkan dengan ISKNV rujukan. Selain itu, analisis filogenetik gen MCP juga menunjukkan bahawa virus daripada genus *Megalocytivirus* boleh dibahagikan kepada tiga genotip: genotip 1 termasuk ISKNV rujukan dan semua jenis strain ISKNV yang dikesan dalam kajian ini. Genotip 2 virus yang berkait rapat dengan red sea bream iridovirus (RSIV), dan genotip 3 virus yang berkait rapat turbot reddish body iridovirus (TRBIV).

Oleh disebabkan tiada tanda-tanda penyakit pada kesemua ikan, terdapat satu kebimbangan atas kemungkinan pemancaran jangkitan ISKNV secara asimptomatik pada spesies ikan hiasan air tawar. Berikutan itu, satu kajian telah dijalankan untuk menyiasat sebaran jankitan ISKNV diantara ram cichlid melalui kaedah kohabitasi. ISKNV telah tersebar daripada ikan yang disuntik kepada ikan kohabitasi dalam masa seminggu dan ikan yang dijangkiti adalah asimptomatik. Jangkitan ISKNV pada ikan telah dikesan dengan menggunakan OIE PCR dan histopatologi. Sel-sel pembawa jasad

rangkuman (IBCs) telah terdapat di hati dan limpa ikan yang dijangkiti. Tambahan pula, perubahan patologi seperti pengumpulan sel-sel inflamasi di pulpa splenium dan peningkatan pusat melano-makrofaj di limpa telah dicerap. Tiada perubahan histologi didapati pada ikan kawalan.

Pemeriksaan petanda klinikal visual tidak sesuai untuk memantau jangkitan ISKNV kerana virus ini mampu wujud sebagai asimptomatik dalam ikan. Oleh itu, kaedah "loop-mediated isothermal amplification" (LAMP) telah dicipta untuk mengesan ISKNV. Empat pencetus telah direka berdasarkan urutan gen protein kapsid utama (MCP) daripada ISKNV. Didapati suhu dan masa optimum untuk kaedah LAMP adalah masing-masing 65 ºC dan 60 minit. Kaedah ini tidak memerlukan apa-apa peralatan canggih dan membenarkan penyiasat untuk menjalankan ujian diagnostik di ladang. Berbanding dengan kaedah diagnosis molekul lain seperti PCR and qPCR, masa tindak balas untuk kaedah LAMP lebih singkat dan mampu memberikan keputusan segera tanpa keperluan prosedur tambahan selepas tindak balas. Pengenalan patogen yang tepat dengan menggunakan alat diagnosis yang khusus adalah penting untuk mengawal penyebaran penyakit berjangkit. Salah satu kelebihan kaedah LAMP ini adalah pengkhususan untuk mengesan ISKNV. Pencetus yang direka adalah khas untuk ISKNV dan tiada sebarang amplifikasi silang berlaku pada red sea bream iridovirus (RSIV), white spot syndrome virus (WSSV), *Aeromonas hydrophila* or *Vibrio parahaemolyticus*. Had pengesanan bagi kaedah LAMP ini adalah 20fg dan acridine orange telah digunakan untuk mengesan kehadiran produk amplifikasi dan keadah novel ini menukarkan warna produk amplifikasi kepada warna kuning untuk menunjuk tindakbalas positif dan warna oren kekal pada tindabalas negatif. Penggunaan acridine orange pada produk LAMP memberikan keputusan kualitatif yang jelas dan boleh dilihat tanpa bantuan lampu khas atau gel agar elektroforesis.

Secara ringkas, tahap jangkitan ISKNV yang termasuk pengetahuan perumah dan taburan geografi di Semenanjung Malaysia telah didedahkan dalam kajian ini. Maklumat ini adalah penting unutk mengurangkan penyebaran penyakit ini. Kajian ini juga menunjukkan sebaran jangkitan ISKNV asimptomatik di ram cichlid secara kohabitasi. Sehingga kini sebaran jangkitan ISKNV asimptomatik di ram cichlid secara kohabitasi tiada dilaporan. Kaedah LAMP merupakan sebuah kaedah diagnostik yang mudah dan menjimatkan kos untuk mengesan ISKNV di makmal dan juga di ladang ternakan ikan.

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I certify that a Thesis Examination Committee has met on 24 July 2014 to conduct the final examination of Kuttichantran a/l Subramaniam on his thesis entitled "Molecular Characterization and Experimental Infection of Infectious Spleen and Kidney Necrosis Virus from Ornamental Fish in Peninsular Malaysia" 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 fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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%	Percentage
ºC	Degree Celsius
$(NH_4)_2SO_4$	Ammonium sulfate
µL	Micro liter
µM	Micromolar
ag	Attograms
ALIV	African lampeye iridovirus
ATPase	Adenosinetriphosphatase
BF	Bluegill fry
bp	Base pairs
CPE	Cytopathic effect
c_t	Cycle threshold
DE	Delayed early
DGIV	Dwarf gourami iridovirus
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscope
EtBr	Ethidium bromide
FEC	Flounder embryonic cell
fg	Femtogram
FITC	Fluorescein isothiocyanate
FV3	Frog virus 3
g	g-force
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSDIV	Grouper sleepy disease iridovirus
h	hour (s)
H&E	Haematoxylin and eosin
HCl	Hydrochloric acid
IAP	Inhibitor of apoptosis protein
IBC	Inclusion body-bearing cell
ICTV	International Committee on Taxonomy of Viruses
IE	Immediate early
IFAT	Indirect fluorescent antibody test
IHNV	Infectious haematopoietic necrosis virus
IKKβ	IκB kinase β
IP	Intraperitoneal
IRAK1	Interleukin-1 receptor activated kinases
IRF	Interferon regulatory factors
ISKNV	Infectious spleen and kidney necrosis virus
KCl	Potassium chloride
KHV	Koi herpesvirus
LAMP	Loop-mediated isothermal amplification
LFD	Lateral flow dipstick

MCIV	Murray cod iridovirus
MCP	Major capsid protein
FF	Mandarin fish fry
$MgSO_4$	Magnesium sulfate
min	minute (s)
mM	Millimolar
n	Sample size
NaCl	Sodium chloride
NF-Kb	Nuclear factor-kappa B
OIE	Office International des Epizooties
ORF	Open reading frame
OSGIV	Orange-spotted grouper iridovirus
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pg	Picogram
PGIV	Pearl gourami iridovirus
qPCR	Quantitative polymerase chain reaction
RBIV	Rock bream iridovirus
rER	Rough endoplasmic reticula
RGV	*Rana grylio* virus
RPS	Relative percentage survival
RSIV	Red sea bream iridovirus
s	second(s)
SBIV	Sea bass iridovirus
sER	Smooth endoplasmic reticula
SG	SYBR Green 1
SKIV	Spotted knifejaw iridovirus
TBIV	Turbot iridovirus
TCID	Tissue culture infective dose
TE	Ethylenediamine tetraacetic acid
TEM	Transmission electron microscope
TF	Turbot fin
TGIV	Taiwan grouper iridovirus
T_m	Melting temperature
TRBIV	Turbot reddish body iridovirus
TSV	Taura syndrome virus
UV	Ultraviolet
VAS	Virus assembly site
WSSV	White spot syndrome virus
zfrIFN1	Zebrafish interferon 1

CHAPTER 1

INTRODUCTION

Infectious diseases are serious threat to the aquaculture industry and the most common infectious agents of fish are viruses, bacteria, fungi and metazoan parasites (Meyer, 1991; Whittington and Chong, 2007). Viruses from the *Iridoviridae* family are reported to cause high mortalities and severe economic losses in the aquaculture industry (Chao *et al*., 2002). According to Williams (1996) and Xeros (1954), Iridovirus was first detected by Claude Rivers in March 1954 and it was isolated from crane fly larvae (*Tipula paludosa*) that glowed with patches of blue coloration. Ever since, more studies on *Iridoviridae* have been initiated and most of the early studies have focused on iridescent viruses (Xeros, 1954). For amphibian iridovirus, early research began from the discovery of an isolate associated with renal carcinoma in the leopard frog (*Rana pipiens*) by Granoff *et al*. (1966).

Infectious iridoviruses have been reported as one of the important causative agents of viral diseases in fish for the past 20 years (Williams *et al*., 2005). These viruses have been reported not only from Europe and America but also widely from East and South-East Asian countries such as Hong Kong, Korea, Malaysia, Philippines, Singapore and Thailand (Chou *et al*., 1998; Mahardika *et al*., 2004; Do *et al*., 2005; Jeong *et al*., 2006).

According to the ninth report of the International Committee on Taxonomy of Viruses (ICTV), the family *Iridoviridae* is subdivided into five genera, *Iridovirus*, *Chloriridovirus*, *Ranavirus*, *Lymphocystisvirus* and *Megalocytivirus* (Jancovich *et al*., 2012). Notably, *Megalocytivirus* has received the most attention in research due to its wide host range and causes significant mortality in the aquaculture industry (Dong *et al*., 2011). The first *Megalocytivirus* was reported from Shikoku, Japan in 1990 (Nakajima and Kurita, 2005) and subsequently in other countries such as Singapore, Korea, Malaysia, China, Australia and Indonesia (Go *et al*., 2006; Song *et al*., 2008).

Infectious spleen and kidney necrosis virus (ISKNV) has been listed as an only species within the genus *Megalocytivirus*. Other viruses such as sea bass iridovirus (SBIV), dwarf gourami iridovirus (DGIV), rock bream iridovirus (RBIV), red sea bream iridovirus (RSIV), Taiwan grouper iridovirus (TGIV), African lampeye iridovirus (ALIV), grouper sleepy disease iridovirus (GSDIV), orange-spotted grouper iridovirus (OSGIV), turbot iridovirus (TBIV) and spotted knifejaw iridovirus (SKIV) are listed as members of this genus but have not been approved as virus species (Jancovich *et al*., 2012).

The ISKNV infects wide range of ornamental fish species such as mandarin fish, *Siniperca chuatsi*; ramirezi cichlids, *Mikrogeophagus ramirezi*; African lampeye, *Aplocheilichthys normani*; murray cod, *Maccullochella peelii peelii*); dwarf gourami, *Colisa lalia*; zebrafish, *Danio rerio*; common platy, *Xiphophorus maculates*; swordtail, *Xiphophorus helleri*; and pearl gourami, *Trichogaster leerii* (Yanong and Waltzek, 2010). In addition, epidemiological study carried out in the South China Sea by Wang *et al*. (2007) have reported that 13 cultured species and 39 wild marine fish species are susceptible to ISKNV-like viruses. In another study, nine out of ten iridoviruses infecting four cultured fish species, namely rock bream, red sea bream, sea bass and rock fish in Korea belongs to members of genus *Megalocytivirus* (Do *et al*., 2005).

Malaysia is one of the major ornamental fish producers among the Asian countries and exported fish worth of US$ 192 million in 2012 (Department of Fisheries Malaysia, 2012). However, the impact and extent of ISKNV infection to the Malaysian ornamental fish industry is unknown at present due to the lack of knowledge on the host range, geographical distribution and the differences between strains if any. In addition, fish which were infected by this virus can be asymptomatic (Jeong *et al*., 2008). Hence, the transmission of asymptomatic ISKNV infection in ornamental fish species has to be investigated in order to take necessary preventive measures to mitigate the spread of this virus.

Diagnostics methods such as histology and transmission electron microscope (TEM) have limited specificity and unable to detect low numbers of the viruses. In addition, PCR and qPCR cannot be carried out in resource limited laboratories and field due to the requirement of sophisticated equipment. A highly specific, simple and inexpensive diagnostic tool is required to detect the presence of ISKNV to monitor the virus to ensure a healthy development of the ornamental fish industry.

Thus, this study was carried out with the following hypotheses and objectives which will be paramount to establish a better understanding of ISKNV that infects farmed ornamental fish from Peninsular Malaysia:

Hypotheses of the study

Null hypothesis

1. There is variation among the ISKNV isolates in Peninsular Malaysia and different ISKNV strains infect different freshwater ornamental fish species
2. Asymptomatic ISKNV infection not transmitted to ram cichlid by cohabitation
3. Designed primers and optimized reaction conditions for LAMP assay unable to detect ISKNV in infected fish

Alternate hypothesis

1. There is no variation among the ISKNV isolates in Peninsular Malaysia and a single ISKNV strain infect a broad range of freshwater ornamental fish species
2. Asymptomatic ISKNV infection transmitted to ram cichlid by cohabitation
3. Designed primers and optimized reaction conditions for LAMP assay are able to detect ISKNV in infected fish

Objectives of the study

1. To detect ISKNV and infer phylogenetic relationship of the isolates based on conserved MCP gene
2. To investigate the transmission of asymptomatic ISKNV infection in ram cichlid by cohabitation
3. To evaluate LAMP assay for rapid and effective detection of ISKNV

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