UNIVERSITI PUTRA MALAYSIA

DETECTION OF PHOSPHOLIPASE C ZETA (PLC-ζ) IN TESTIS OF Rattus argentiventer Robinson & Kloss (RICE-FIELD RAT) USING MOLECULAR TECHNIQUES

FADZLINA BINTI AMIR SHAPUDDIN

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By

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Thesis Submitted to the School of Graduate Studies,
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May 2013

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Abstract of thesis presented to the Senate of University Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

DETECTION OF PHOSPHOLIPASE C ZETA (PLC- ζ) IN TESTIS OF *Rattus argentiventer* Robinson & Kloss (RICE-FIELD RAT) USING MOLECULAR TECHNIQUES

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May 2013

Chair : Associate Professor Sabrina Sukardi, PhD
Faculty: Medicine and Health Sciences

Phospholipase C-zeta (PLC-ζ) is a specific enzyme found in sperm of mammals responsible for triggering calcium oscillations leading to oocyte activation during fertilization. The activation causes the fertilized oocyte to divide and develop into an embryo. *Rattus argentiventer* (Rice Field Rat) is often responsible for destruction of agricultural crops specifically paddy fields. The mammal is known for its rapid reproductive potential and as a carrier of pathogen. This rodent pest population is currently being controlled with baits, traps and biological control such as *Tyto alba*. However the current methods are quite expensive, needs long term monitoring and are hazardous to the environment. Thus the knowledge of fertility such as sperm factor of this rodent could be helpful in a way to prevent overpopulation of this rodent. Two detection methods to identify PLC-ζ gene fragments from the testis of *Rattus argentiventer* using conventional Polymerase Chain Reaction (Reverse Transcriptase RT-PCR) and Real Time Polymerase Chain Reaction (qRT-PCR) techniques were used *in* order to obtain the bands of PLC-ζ. Following that, sequencing of DNA structure and cloning of PLC□ were performed to amplify gene fragment of PLC-ζ. Approximately 420 bp nucleotides identified sequence was then keyed in into the Basic Local Alignment Search Tool (BLAST) portal in National Centre of Bioinformatics Information (NCBI) for comparison with standard nucleotide sequences from other species for sequence alignment. The specific target 420 bp nucleotides of PLC-ζ were cloned using a kit through ligation and transformation. The result showed that PLC-ζ enzyme was present in *Rattus argentiventer* using PCR technique. However, the cloning procedure carried out was not able to show the presence of target gene fragments of the enzyme. It is confirmed that PLC-ζ enzyme was present in *Rattus argentiventer* through the two detection

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methods used. The study is a novel study to detect of PLC-ζ in paddy rats using molecular approaches. The result obtained with RT-PCR was validated with qRT-PCR and the sequence obtained showed excellent similarity homology with published sequence of PLC-ζ in *Rattus norvegicus*. As such this would give valuable baseline information for future researches to be carried out in the approach of controlling and preventing the continous growth of *Rattus argentiventer* population.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGESANAN PHOSPHOLIPASE C ZETA (PLC- ζ) DALAM TESTIS *Rattus argentiventer* Robinson & Kloss (TIKUS SAWAH) MENGGUNAKAN KONVENSIONAL REVERSE TRANCRIPTASE (RT-PCR) DAN *REAL TIME* POLYMERASE CHAIN REACTION (qRT-PCR)

Oleh

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Phospholipase C-zeta (PLC-ζ) adalah enzim spesifik yang ditemui di dalam sperma mamalia yang bertanggungjawab mencetuskan ayunan kalsium menyebabkan pengaktifan oosit semasa persenyawaan. Dengan pengaktifan ini oosit yang tersenyawa membahagi dan berkembang menjadi embrio. *Rattus argentiventer* (Tikus Sawah Padi) sering menyebabkan kemusnahan tanaman pertanian khususnya sawah padi. Mamalia spesis ini berpotensi membiak dengan cepat dan pembawa patogen. Tikus perosak ini biasanya dikawal dengan umpan, perangkap dan kawalan biologi contohnya dengan menggunakan perangkap *Tyto alba*. Walau bagaimanapun, kaedah semasa yang digunakan adalah agak mahal, memerlukan pemantauan jangka panjang dan berbahaya kepada alam sekitar. Oleh itu, pengetahuan mengenai faktor kesuburan seperti sperma tikus ini boleh membantu dalam cara untuk mengawal pembiakan yang terlalu tinggi. Dua kaedah pengesanan untuk mengenal pasti gen PLC-ζ dari testis *Rattus argentiventer* adalah menggunakan *Reverse Transcriptase* (RT-PCR) dan *Real Time Polymerase Chain Reaction* (qRT-PCR) dalam usaha untuk mendapat PLC-ζ nukleotida. Dengan itu, penjujukan struktur DNA dan pengklonan PLC-ζ telah dilakukan untuk mengenali struktur DNA gen PLC-ζ. Kira-kira 420 bp nukleotida yang dikenalpasti kemudiannya dimasukkan ke dalam portal BLAST di *National Centre of Bioinformatics Information* (NCBI) untuk perbandingan dengan spesis lain. Sasaran 420 bp PLC-ζ nukleotida diklon menggunakan kit melalui proses ligasi dan transformasi. Hasilnya menunjukkan bahawa enzim PLC-ζ hadir dalam *Rattus argentiventer* menggunakan kaedah PCR. Walau bagaimanapun, prosedur pengklonan tidak dapat menunjukkan kehadiran enzim PLC-ζ. Dua kaedah kajian pengesanan menggunakan pendekatan molekular mengesahkan bahawa enzim PLC-ζ hadir dalam testis *Rattus*

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argentiventer. Hasil yang diperolehi dengan RT-PCR telah disahkan dengan qRT-PCR dan urutan yang diperolehi menunjukkan persamaan homologi tikus piawai PLC-ζ yang telah dikenalpasti. Maklumat yang diperolehi ini akan memberikan panduan asas yang sangat berharga untuk kajian akan datang dengan pendekatan mengawal dan mencegah populasi pembiakan tikus yang tinggi daripada spesis *Rattus argentiventer*.

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APPROVAL

I certify that an Examination Committee has met on 15 May 2013 to conduct the final examination of Fadzlina Amir Shapuddin on her Master of Science thesis entitled "Detection of Phospholipase C Zeta (PLC- ζ) In Testis Of *Rattus argentiventer* Robinson & Kloss (Rice-Field Rat*)* Using Molecular Techniques" in accordance with Universities and Universitiy College Act 1971 and Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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

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LIST OF ABBREVIATIONS

A	Adenine
A260	Absorbance at 260 nm
A280	Absorbance at 280 nm
Amp	Ampicillin
APC	Anaphase promoting complex
BAPTA	*N*,*N*-(1,2-ethanediylbis(oxy-2'l-phenelyne)bisN(carboxymathyl))-glysine
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C	Cytosine
Ca^{2+}	Calcium
CaMKII	Calmodulin-dependent protein kinase
CAMP	Cyclic adenosine monophosphate
cDNA	Complemetary deoxyribonucleic acid
Ct	Cycle threshold
DAG	Diacylglycerol
DdNTPs	Dideoxynucleotide triphosphates
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
EST	Express sequence taq
EtOH	Ethanol
FA	Formaldehyde Agarose
Fg	force exerted by gravity
FSH	Follicle Stmulating Hormone
G	Guanine
g	Gram
GPI	Glycosylphosphatidyl-inositol
ICSI	Intracytoplasmic Sperm Injection
IP3	Inositol triphosphate
IPTG	Isoprapnol β-D-galactopyranoside
IVF	In vitro fertilization
L	Litre
LB	Luria-Bertani
Mg	Miligram
MIQE	Minimum Information for Publication of Quantitative Real Time PCR Experiments
Mins	Minute
ml	Millilitre
mM	Millimolar
MOPS	3-(N-morpholino) propanesulfonic acid
MW	Molecular weight
NA	Nutrient Agar
N	Nucleus

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NaCl	Sodium chloride
NCBI	National Centre of Bioinformatics Information
Nm	Nanometer
ºC	Degree celcius
OD	Optical density
PAGE	Polyacrymide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PD	Plasmid DNA
PIP2	Phosphotidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC- ζ	Phospholipase C zeta
PN	Pronucleus
qPCR	Relative quantitative real-time polymerase chain reaction
RA	*Rattus argentiventer*
RNA	Ribonucleic acid
rpm	Revolution per minute
RT-PCR	Reverse trancriptase polymerase chain reaction
S	Svedberg
SF	Sperm Factor
SYBR	Sybergreen
T	Thymine
TBE	Tris Borate
Tm	Melting temperature
V	Volt
X-gal	5-bromo-4chloro-3-indolyl-β-D-galactopyranoside
β-ME	β-mercaptoethanol

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Phospholipase C zeta (PLCζ) is an enzyme which induces a surge of calcium in the fertilized ovum causing it to divide and develop into an embryo. This enzyme is found in the sperm which triggers calcium oscillations leading to oocyte activation during fertilization. Oocyte activation is an important biological process that allows the release of mammalian oocytes which are arrested at the metaphase of the second meiotic division (MII). Saunders et al., (2002) uses mouse express sequence tag databases and discovered a novel, and importantly testis-specific, Phospholipase C (PLC), termed PLC zeta (PLCζ), which is a protein of ~74 kDa in mice and ~70 kDa in humans. This is the smallest PLC isoform and is found to play an important part in mammalian oocyte stimulation and demonstrate that immunodepletion of PLCζ from sperm extracts suppresses Ca^{2+} releasing ability whereas sperm studies have indicated that the presence of PLCζ in sperm correlates with the sperm's ability to induce Ca^{2+} oscillations in the oocyte (Fujimoto et al., 2004; Kurokawa et al., 2005). Jones et al., (2000) and Jones, (2005) demonstrated that PLCζ has been involved in the release of this arrest as a physiological agent of oocyte activation. Therefore it is found that PLCζ is the physiological agent of mammalian oocyte stimulation (Swann et al., 2006; Whitaker, 2006; Parrington et al., 2007; Kashir et al., 2010). PLCζ mRNA was found to exist as early as day 17 (Young et al., 2009) in nothern blot analyses of testes from postnatal hamsters. It is predominantly localized to post-acrosomal parts of the sperm head in mice, a pattern preserved before the acrosome reaction in immunofluorescence studies (Fujimoto et al., 2004; Yoon & Fissore, 2007; Young et al., 2009). It has been shown to be a potential target in manipulating fertility of male mammals. One of the molecular approaches that can be used is silencing the sperm factor known as Phospholipase C-zeta (PLC-ζ), an enzyme that is involved in fertilization (Swann et al., 2004). In this study Conventional Reverse Tranciptase PCR (RT-PCR) Polymerase Chain Reaction is the molecular approach chosen to detect sperm-specific factor PLC-ζ in rodents pest namely *Rattus argentiventer*. These rodent pests are a rapid breeder and a carrier of pathogens. The method is based on the study conducted by Fujimoto in 2004 who designed the primary sequence for PLCζ in rodents. Real Time PCR (qRT-PCR) was used to detect and validate the result obtained from RT-PCR using the same primers.

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1.2 Problem Statements

Rice is a staple food for the Asean region. Rat infestation can cause paddy yield to decrease. *Rattus argentiventer* known as Rice Field Rat (Robinson and Kloss, 1918) was normally cought at paddy fields. These rats are serious pest of paddy and plantation such as oil palm and cocoa (Lam, 1982). The rice field rat is one of the rodent species that is responsible for destructions of all stages of paddy growth in South East Asia such as Malaysia (Wood & Fee, 2003). Precautions need to be implemented at each stage of the paddy growth in order to avoid destruction by these pest which are time consuming and costly. This rodent breeds during the reproductive phase of the rice crop (Lam, 1983). They breed rapidly and could reach high population density in a very short time. Every single breeding pair is able to produce 600 offspring in three months (Vail, 2008). A few studies in Malaysia showed that *Rattus argentiventer* is a carrier of pathogens that could be harm to humans even though the percentage carried is lower compared to other pests studied in Malaysia. These parasite includes intracellular parasites such as ectoparsite, nematodes and protozoa (Shafiyyah et al., 2012). Currently, rodent pest populations are controlled by rodenticides such as warfarin (Buckle et al., 1984) baits, traps (an upgrade of traps combination with physical barrier known as –TBS) and biological methods using owls such as Tyto alba. The excessive use of anticoagulant rodenticides such as vitamin K antagonist that disrupt normal blood clothing mechanism causing lethal haemorrhage resulted in the animals developing resistance to the lethal effects. In addition, improper use of baits could be lethal to non-target animals. Residues of rodenticides found in dead or dying rodents could also be toxic to scavengers and predators (Hoare & Hare, 2006). A recent approach controlling rice field rat populations based on fertility control was by using chemical sterilant that accelerates the natural reproductive ageing process in the rat resulting in sterility or reproductive 'senescence' known as 'contrapest' while removing the adverse effect of poisons on non-target species (Vail, 2008).

As PLCζ has been shown to be a potential target in manipulating the fertility of male mammals (Swan et al., 2004), therefore the aim of this study was to isolate and identify the presence of PLCζ in *Rattus argentiventer* testis. It is hope with the discovery of PLCζ from this rodent could further propagate more research in this area for future potential in developing new strategies in pest control.

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1.3 Objective

1.3.1 General objective

To identify Phospholipase C-zeta (PLC-ζ) in *Rattus argentiventer* (Rice Field Rat).

1.3.2 Specific Objectives:

1. To isolate and amplify PLC-ζ gene fragment from testis of *Rattus argentiventer*.
2. To validate PLC□ gene fragment obtained from conventional PCR with qRTPCR.
3. To determine the sequence of PLC-ζ *Rattus argentiventer* and compare the sequence of PLC-ζ *Rattus argentiventer* with published PLC-ζ sequences.
4. To clone the PLC-ζ sequence.

1.4 Research Hypothesis

PLC-ζ enzyme is present in testis of *Rattus argentiventer* using conventional Reverse Transcriptase (RT-PCR) and Real Time (qRT-PCR) techniques.

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