IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN: 2278-3008, p-ISSN:2319-7676. Volume 8, Issue 3 (Nov. – Dec. 2013), PP 65-74
www.iosrjournals.org

Detection of PLC-ζ From Testis of *Rattus Argentiventer*(Rice-Field Rat) Using RT-PCR and qRT-PCR

A.S.Fadzlina, A.M.N. Nasyriq, M.N. Noor Faezah, L.H. Lee, Y.K. Cheah and S. Sukardi

Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, UPM Serdang, 43400, Selangor.

Abstract:*Rattus argentiventer (Rice Field Rat) is responsible for destruction of paddy. The mammal is known for its rapid reproductive potential which can be a target for biological control of this species. Phospholipase C-zeta (PLC ζ) is a specific enzyme found in sperm of mammals responsible for triggering calcium oscillationsleading to egg activation during fertilization. It facilitates the first step of egg activation causing egg division and subsequent development into an embryo. The method used in this study was to identify PLCζ gene fragments from the testis of Rattus argentiventer using two-step Reverse Transcriptase Polymerase Chain Reaction (PCR) and quantified with Real Time Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR). Following that, sequencing of PLCζ was performed. The identified sequence was then keyed in into the BLAST portal in NCBI (National Centre of Bioinformatics) for comparison with a standard PLCζ sequence from Rattus norvegicus for sequence alignment. The result showed that PLCζ was present in Rattus argentiventer and qRT-PCR could quantify the amount of PLCζ available.As such this would give additional information of detection and quantification techniques for rapid identification and detection of PLCζ to be carried out in the approach of controlling the rapid growth of Rattus argentiventer population.*

Keywords:*Phospholipase C-zeta (PLCζ),Rattus argentiventer, testes, RT-PCR, qRT-PCR*

I. Introduction

Rodents breed rapidly and can reach high population density across all stages of growth of paddy. The rapid reproductive potential of this species may lead to uncontrolled population growth causing destruction of paddy.*Rattus argentiventer* (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). Precaution need to be implemented at each stage of the paddy growth in order to avoid paddy from being destroyed by the rodent.Currently this rodent pest population is being controlled by rodenticides such as warfarin (Buckle et. al., 1984) or baits, traps and biological control such as the barn owl *Tyto alba*. Rodenticides were used excessively until the rodent developed resistance to their lethal effects. In addition, the misuse of these baits could be lethal to non-target animals. Residues of rodenticides found in dead or dying rodents could also cause toxic effects to existing scavengers and predators(Christensen, et. al., 2012).As a result, the present pest controlling methods are found to be non-cost effective, needs long term monitoring and hazardous to the environment.

A biological method of pest control has become increasingly popular because they are safe and cost effective (Wood and Fee, 2003). The study of the reproductive stage of the rodent using molecular approach may contribute to the foundation of developing biological methods of pest control. One of the molecular approaches suggested to prevent overpopulation of this pest is by controlling the sperm factor known as Phospholipase C-zeta (PLCζ) that is involved in fertilization. PLCζ is a specific enzyme found in sperm which triggers calcium oscillationsleading to egg activation during fertilization. The enzyme induces a surge of calcium levels in egg and later causes eggs to divide and develop into an embryo (Saunders,et al., 2002).PLCζ has been shown to be a potential target in manipulating the fertility of male mammals (Swann, et. al., 2004). This can be one of the biological methods to interrupt the embryonic development. The possible mechanism of this method includes initiation of short-interference RNA (siRNA) into living cells or mammalian cultured cells. This technique has been shown to enable control of the growth of cancer cells (Tebes & Kruk, 2005). The initiation of siRNA would interrupt the expression of PLCζ in these rodents by silencing the gene responsible to express the protein. This may in turn help to control male fertility, inhibit fertilization thus controlling the rodent population.

PLCζ has been detected in the testes of *Rattus argentiventer* using two-step RT-PCRby Jaffar et. al.,in 2010. This study is a continuation of the previous study with the addition of qRT-PCR as another technique to identify and quantify PLCζ. It is hope that with the addition of another detection technique, detection of this enzyme in samples other than rodents could be establishand more rapid detection and quantification of the amount of PLCζ available in samples could be easily carried out.

II. Material and Methods

2.1 Collection of Samples in Control Pest Centre Malaysian Agriculture Research and Development Institute (MARDI) Bumbung Lima, Pulau Pinang, Malaysia.

The rodents were collected in conventional rodent traps. The traps were placed overnight during each stage of the paddy growth such as tillering, boosting and harvesting. The cages were monitored daily until rodents were trapped. The rodents were placed in a cage and later transferred to a high drum to prevent escape.*Rattusargentiventer* was differentiated from other species by the presence of a white belly and twelve nipples. Precautions were needed to handle these rodents as they were wild and dangerous. The rodents were then transferred to another cage and moved to the study location. The rodents were kept and maintained in cages at the Animal House, Faculty of Medicine and Health Sciences, UPM. They were fed three times daily with young paddy or pellet and water.

2.2 Preparation of Samples

The rodents were sacrificed with chloroform inhalation. Thetestis were dissected out and placed in distilled water to flush it form debris prior to flash-freezing in liquid nitrogen. Immediately after that, the frozen testes were ground quickly using a pestle and mortar until it became powder. The end product was weighed. The samples were then subjected to RNA extraction using a standard procedure according to (Yoneda et al., 2006; Mizushima et al., 2009;Young, et. al., 2009) then an isolation process was perform using easy-BLUE^{TM} Total RNA Extraction Kit (Intron Biotechnology, Korea).

2.3 RNA Extraction

Approximately 100 mg of the fresh sample was placed into a 2.0 ml centrifuge tube. Approximately 1 ml of easy-BLUE^{TM} reagent (Intron Biotechnology, Korea) was added to the sample and then homogenized. The mixture was then vortex vigorously for 10s until no clumps were seen. Approximately 100 µl of chloroform was then added to the mixture to separate the phenol layer from the aqueous layer and eventually the RNAwas isolated. After vortexing the mixture, it was then centrifuged at 13,000 rpm at 4°C for 10 min. Approximately 400µl of the upper fluid level was then transferred to an empty 1.5 ml tube. Three layers (upper aqueous layer, middle white layer and lower phenol layer) were formed after addition of chloroform. The upper aqueous layer contained RNA and white sediments were formed at the base of the lower phenol layer. These white sediments consist of mixtures of protein and genomic DNA. The lower phenol layer (blue colour) contained denatured protein or cell debris. Subsequently, 400µl of isopropanol (2-propanol) was added into the tube containing the transferred upper fluid level and the mixture was then mixed by inverting the tube 2 to 3 times. The mixture was then left for 10 min at room temperature. Formation of a white layer which contained RNA could be observed after adding isopropanol. After 10 min, the mixture was then subjected to centrifugation at 13,000 rpm at4°C for 5 min and the upper layer was discarded to obtain the RNA pellet. Approximately 1 ml of 75% ethanol was added to the white RNA pellet and the solution was mixed well by inverting the tube 2 to 3 times. Later, the mixture was centrifuged for 5 min at 10,000 rpm at 4°C. The upper layer was discarded and the remaining RNA pellet was left to dry. The RNA was then dissolved using 20 to 50µl of DEPC treated distilled water. The purified RNA was stored at -80^0C for long-term or at -20^0C for short term storage.

2.4 Quantification of RNA

The RNA was quantified using a spectrophotometer (Eppendorf Biophotometer, Hamburg, Germany). The concentration of RNA was determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. The spectrophotometer was blanked at 260 nm with ultrapure distilled water. Appropriate dilution of the RNA sample was prepared. The conversion factor for RNA was 40 µg/µl per OD_{260} unit. The spectrophotometer reading was recorded. For a reading of 0.10, the concentration was calculated as follows:

$$\text{Concentration} = A_{260} \text{ x dilution factor x conversion factor}$$

The spectrophotometer was blanked at 280 nm with water or buffer. The optical density (OD) of the mRNA sample was measured at 280 nm. The ratio between the absorbance values at 260 nm and 280 nm gave an estimation of the RNA purity. Pure RNA has an A_{260}/A_{280} ratio of 1.8 to 2.1. Lower ratios indicate possible protein contamination, or low pH in the solution used as a diluents for spectrophotometer readings.

2.5 Integrity of RNA

This method was carried out to observe the integrity of RNA.Approximately 0.18 g of agarose powder was weighed and mixed with 1.5 ml 10X Formaldehyde to form the formaldehyde agarose (FA) gel. The mixture was then heated for about one min until the agarose had dissolved. The agarose was allowed to cool to 65 to 70°C under running tap water. Approximately 275 ml of 37% formaldehyde was added to the mixture and

poured onto a rectangular casting tray containing a comb with 12 wells. The gel was left to settle for at least 30 min. After the gel has solidified, the comb was carefully removed and the gel was placed in the electrophoresis tank filled with 1X FA gel. The gel was soaked in the 1X FA buffer at approximately one mm of it surface. The gel was then left to equilibrate in the buffer for at least 30 min. One volume of 5X RNA loading buffer was mixed with 4 volumes of RNA sample. The mixture was then incubated for 5 min at 65°C and chilled on ice for about 3 min before the loading process took place. Once samples were loaded, electrophoresis was run at a constant voltage of 70V for 40 min. The gel was stained with ethidium bromide for 5s and destained under running tap water for another 10 min. The bands in the gel were observed under UV light using Alpha Imager™ 2200 (Alpha Innotech Corporation, South Africa). The next step was to convert RNA to cDNA using reverse transcriptase enzyme. The cDNA was used as a template to amplify DNA using conventional PCR and Real-Time PCR to identify the target gene.

2.6 Two step-Reverse Transcriptase Polymerase Chain Reaction(RT-PCR)-cDNA Synthesis

The process of RNA conversion to cDNA began with mixing of mRNA, Oligo$(dT)_{18}$ and primers into Tetro cDNA Kit (Bioline,USA). The kit contained MMLV Reverse Transcriptase enzyme to synthesize cDNA. At the initial stage, two tubes were labelled as priming premix and reaction premix. Priming premix contained 3.0 µlof total RNA, 0.5 µl of forward primer, 0.5 µl of reverse primer 1.0 µl ofOligo$(dT)_{18,}$ 1.0 µl of 10 mM dNTP mix and 4.0 µl of DEPC-treated water which gave a total volume of 10 µl.The priming premix solution were prepared on ice and incubated at 70°C for five min. Immediately after 5 min incubation, it was transferred and chilled on ice for 1 min.

Meanwhile, the reaction premix solutions which contained approximately 3.0 µl of 5x RT Buffer, 1.0 µl of Ribosafe RNase Inhibitor, 1.0 µl of Tetro Reverse Transcriptase, 5.0 µl of DEPC-treated water were prepared.All components in the reaction premix tube were added to the priming premix tube and mixed gently. The mixture was first incubated at 45°C for 30 min in the water bath. Later it was transferred to another water bath and incubated at 85°C for 5 min to terminate the reaction. Immediately after that it was transferred and chilled on ice. The synthesized cDNA was later used as a template to amplify DNA using PCR process. Prior to the process of DNA amplification, the temperature of annealing was optimized.

2.7 Optimization of temperature using conventional PCR

Twelve tubes containing 21.0 µl of RNase-free water, 25.0 µl of MyFi™Mix 2x (Bioline, USA), 1 µl of forward primer, 1 µl of reverse primer and 2.0 µl of cDNAwhich gave a total volume of 50 µl volume of mixture solution were prepared. A specific primer with a pair nucleotide of PLCζforward 5'-CATGTGAAACATATTTTTAAGGAAA-3' and PLCζreverse5'-ATCCCCAAATGTCACTCGGTCC3' (Fujimoto et al., 2004) were used to amplify cDNA. All the tubes were labelled and positioned in a gradient thermal cycler (Eppendorf, Germany). The temperature for initial denaturation was initially set at 95°C for 1 min then followed by denaturation at 94°C for 15 s. The gradient temperature for annealing was optimized from 50°C to 60°C for 15 s and the temperature for extension process occurred at 72°C with 35 cycles. This optimization process produced a gradient of PCR products and they were analysed using agarose gel electrophoresis. The band size of the product was determined according to a reference DNA ladder (Bioline, USA).

2.8 Amplification of cDNA, Conventional PCR

The conventional PCR method was used in the amplification of DNA. This method began with a process of mixing 21.0 µl of RNase-free water, 25.0 µl of MyFi™Mix 2x (Bioline, USA), 1 µl of forward primer, 1 µl of reverse primer and 2.0 µl of cDNA in a tube which gave a total volume of 50 µl volume of mixture solution. A specific primer with a pair nucleotide of PLCζforward 5'-CATGTGAAACATATTTTTAAGGAAA-3' and PLCζreverse 5'-ATCCCCAAATGTCACTCGGTCC-3' (Fujimoto et al., 2004) were used to amplify cDNA. β-actinforward 5'-GTTTGAGACCTTCAACACCC-3' and β-actin reverse 5'-CCAATGGTGATGACCTGGCC-3' as a control (Carmosino, 2005) served as a positive control in the amplification process. The mixture was then kept in the thermal cycler machine (Eppendorf, Germany) for denaturation process. Based on PCR cycling conditions, the initial denaturation was set at94°C for 15s followed with denaturation at 94°C for 15s, annealing at 49°C for 15s and extension at 72°C with 35 cycles. The PCR products (amplicons) was then run on gel electrophoresis for band observation.

2.9 Identification of PLCζ, Agarose Gel Electrophoresis

This procedure was carried out to identify the presence of PLCζ gene.The amplicon DNA fragments were separated and analysed using agarose gel electrophoresis. In the preparation of 1.5% agarose gel, 0.3 g of agarose was dissolved in 20 ml of 1X TBE buffer and the mixture was heated until dissolved. The mixture was

left to cool at about 55^0C before pouring it into a rectangular casting tray containing a comb with twelve wells. The gel was then allowed to solidify at room temperature for about 30 min.The comb was gently removed to prevent spoiling of the gel. Approximately 20 µl of amplicons from the conventional PCR process were loaded into eight wells of the gel. A mixture of approximately 1 µl of 1 kB ladder (Bioline, USA), 1 µl of loading dye and 4 µl of buffer was loaded in one of the empty wells as a DNA marker. Then the tray was placed into the electrophoresis chamber that was already filled with 1X TBE buffer. The electrophoresis chamber was connected to the electrodes accordingly and was run at a constant voltage of 70 V for 45 minutes. Following electrophoresis, the gel was stained with ethidium bromide for 5 s and destained with running tap water for another 10 min before examining it under UV light using Alpha Imager™ 2200 (Alpha Innotech Corporation, South Africa). The product of this process was then compared with amplicons produced by Real-Time RT-PCR (qRT-PCR) for validation of the result according to MIQE guidelines.

2.9.1 Validation of PLCζ, gene fragment, Real-Time RT-PCR (qRT-PCR)

The qRT-PCR method was performed to validate the gene fragments of PLCζ enzyme produced by conventional PCR. The extracted RNA from the samples was used in this experiment. The process used Master Mix qRT-PCR (Qiagen, USA) to synthesize cDNA in the Mini Opticon Thermal Cycler (Biorad, USA) quantitative PCR.The reaction mixture of 25 µl volume was prepared in a single tube and it contained 12.5µl of 2x Quantifast SYBR Green RT-PCR Master Mix, 1 µlof forward primer, 1 µl of reverse primer, 0.25 µl of Quantifast RT mix, 9.25 µl of RNase-free water and 1 µl of template mRNA ($\leq$ 200 ng/reaction). The solution was mixed thoroughly and appropriate volumes were dispensed into the PCR vessels. The PCR vessels were placed in the Mini Opticon real-time thermocycler (Biorad, USA). The protocol and the plate were set up then started based on the following real-time cycler conditions; reverse transcription at 50°C for 10 min, PCR initial activation step at 95°C for 5 min, two-step cycling denaturation at 95°C for 10 s, combined annealing and extension at 49°C for 30 s, number of cycles was 35 at 95°C, melting at 72°C for 5 s and 90°C for 8 s and cooling a 40°C for 30 s. The samples were arranged accordingly as shown in figure 3.5.1. β-actin was designated as a positive control in the process. The result was then validated.

2.9.2 Gel Extraction

Gel extraction was performed to excise and purify the desired cDNA fragmentsfrom the agarose gel by using HiYield Gel/PCR DNA Fragments Extraction Kit (Taiwan). The exact size of the band containing cDNA fragments was excised using a scalpel. Approximately 300 mg of the gel slices were transferred into a 1.5 ml microcentrifuge tube followed by adding 500 µl of buffer into the sample and subsequently vortex. The samples were then incubated at 55°C in a water bath for 10 to 15 min until the gel slices have completely dissolved. During incubation, the tubes were inverted every 2 to 3 min. A column was placed in a collection tube and 800 µl of the sample mixture from the previous process was pipetted into the column. The samples were then centrifuged at 8000 rpm for 30 s, flow-through was discarded and the column was placed back in the collection tube. Approximately 500 of wash buffer was added into the column and centrifuged at 8000 rpm for 30 s. The flow-through was discarded and the column was placed back in the collection tube. The step was repeated at least twice before drying the column matrix. The dried column was then transferred into a new microcentrifuge tube. Approximately 15 ml of elution buffer or water was added in the centre of the column matrix and was allowed to stand for about 15 min until the elution buffer was absorbed by the matrix before spinning for 2 min at full speed to obtain purified DNA.

2.9.3 Sequencing of PLCζ at First BASE Laboratories

This method was carried out todetermine thenucleotide sequence, label and separateDNA fragments to a singlenucleotide. Sequencing of PLCζ gene was carried out at First BASE Laboratories, Malaysia using DNA automated sequencer. There were a few steps involved before out sourcing to First BASE Laboratories. The purified DNA fragments of PLCζ were initially transferred to a 0.2 ml centrifuge tube and kept at -20°C. The application form required by the company was filled up manually and at the same time the online application request was submitted. The respective company responded within 24 hours and collected the samples. The samples were then delivered by a courier service to First-BASE laboratories for sequencing assay. The result was informed via electronic mail within two days after delivery and subsequently it was keyed into the NCBI BLAST database to compare with a standard nucleotide sequence from other species for sequence alignment. The result was also examined using 'Finch TV' chromatogram software.

III. Results

3.1 Integrity of RNA

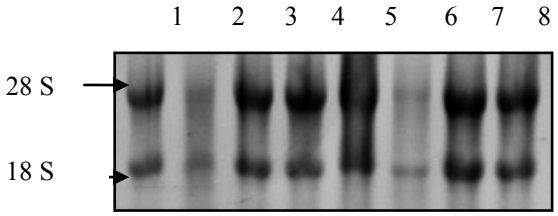


Figure 1: Sample integrity via denaturing formaldehyde agarose. Lane 1 to lane 8 represents eight strains of RNA samples of Rattus argentiventer. Two distinct ribosomal RNA bands (18S and 28S) were seen.

Integrity of RNA was measured using denaturing formaldehyde agarose gel as depicted in Figure 1. Lanes 1 to 8 represents eight strains of RNA samples of *Rattus argentiventer*. Two distinct ribosomal bands at 18S and 28S were observed confirming the integrity of the RNA in the samples.

3.2 Optimization of temperature using conventional PCR

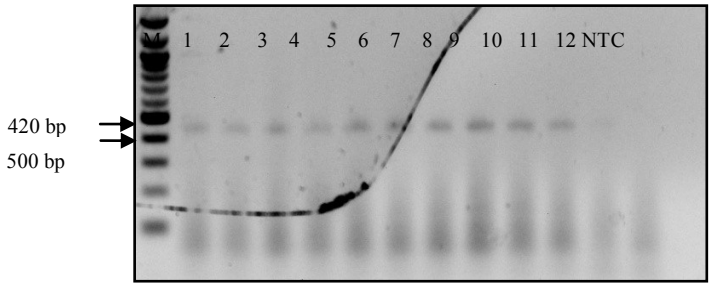


Figure 2: Gel electrophoresis of gradient PCR using cDNA sample forward and reverse primer. Lane M; 1Kb bp DNA ladder, Lane 1; annealing at 39 ^{o}C, Lane 2; annealing at 39.2 ^{o}C, Lane 3; annealing at 40.3 ^{o}C, Lane 4; annealing at 42.1 ^{o}C, Lane 5; annealing at 44.4 ^{o}C, Lane 6; annealing at 47.6 ^{o}C, Lane 7; annealing at 49.7^{o}C, Lane 8; annealing at 52.5^{o}C, Lane 9; annealing at 55.0^{o}C, Lane 10; annealing at 57.2 ^{o}C, Lane 11; annealing at 58.8^{0}C: Lane 12; annealing at 59.6^{0}C. NTC; non-template control

Figures 2 depict the results from gel electrophoresis of gradient PCR using cDNA forward and reverse primers. Different annealing temperature was used to find the optimum temperature for maximum RNA recovery. The range of annealing temperature which showed the maximum intensity of bands was from 44.4 to 57.2 ^{o}C.

3.3 Amplification of PLCζ

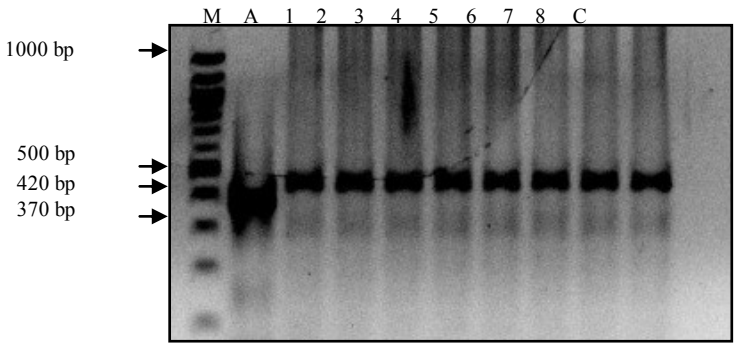
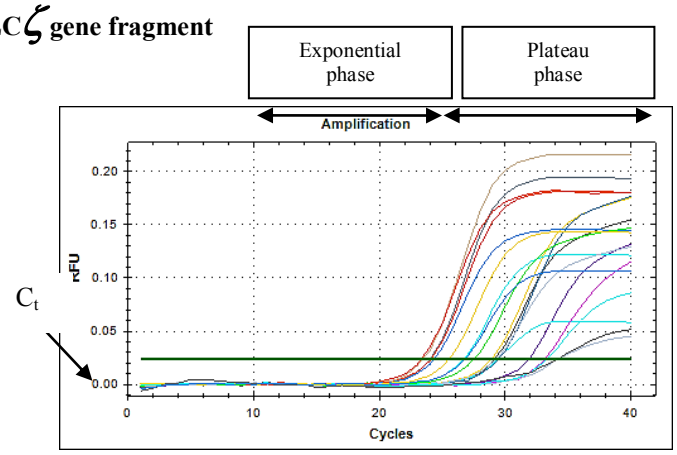


Figure 3:RT-PCR amplification PLCζ from Rattus argentiventer testes. Lane M; 1Kb bp DNA ladder, Lane A; β-actin, Lane 3-lane 8, eight cDNA samples from eight different strain of Rattus argentiventer, Lane 9; Control

Figure 3 depict the amplification of PLCζ from RT-PCR conducted.β-actin was used as a positive control. Lanes 3 to 8 showed clear bands of PLCζ at 420 bp.

3.4 Validation of PLCζ gene fragment



PLCζ

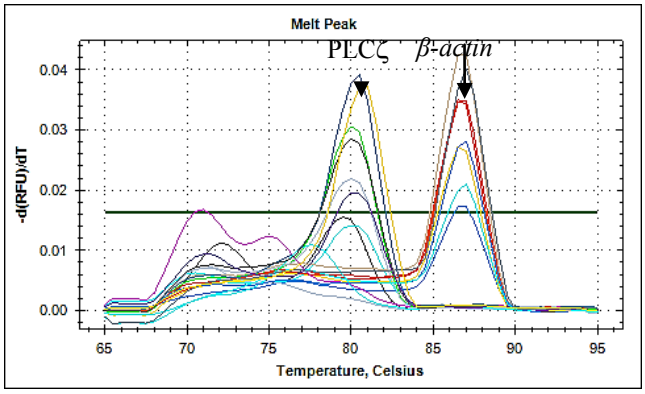
RA1 RA2 RA3 RA4 RA5 RA6 RA7 RA8

β-actin

RA1 RA2 RA3 RA4 RA5 RA6 RA7 RA8

Figure 4: Amplification curve for *Rattus argentiventer* RA1-RA8

Figure 4 showed the validation of PLCζ gene fragments using Real Time RT-PCR (qRT-PCR).



PLCζ

RA1 RA2 RA3 RA4 RA5 RA6 RA7 RA8

β-actin

RA1 RA2 RA3 RA4 RA5 RA6 RA7 RA8

Figure 5: Melt peak for both PLCζ and β-actin

Figure 5 shows the melt peak for both PLCζ and β-actin while Table 1 depicts the melt curve data of qRT-PCR amplification of PLCζ from *Rattus argentiventer*.

Table 1: Melt curve data of RT-PCR amplification PLCζ from*Rattus argentiventer* RA1-RA8.

Well	Fluorescence	Target	Content	Sample	Melt temperature
A01	SYBR	PLCz RA1	Unkn	RA1	81.00
A02	SYBR	β-actin RA1	Unkn		87.00
B01	SYBR	PLCz RA2	Unkn	RA2	80.00
B02	SYBR	β-actin RA2	Unkn		87.00
C01	SYBR	PLCz RA3	Unkn	RA3	80.00
C02	SYBR	β-actin RA3	Unkn		87.00
C03	SYBR	β-actin RA	NTC		None
D01	SYBR	PLCz RA4	Unkn	RA4	80.50
D02	SYBR	β-actin RA4	Unkn		87.00
D03			Neg Ctrl		None
E01	SYBR	PLCz RA5	Unkn	RA5	80.00
E02	SYBR	β-actin RA5	Unkn		86.50
E03			NTC		None
F01	SYBR	PLCz RA6	Unkn	RA6	80.00
F02	SYBR	β-actin RA6	Unkn		86.50
G01	SYBR	PLCz RA7	Unkn	RA7	None
G02	SYBR	β-actin RA7	Unkn		86.50
H01	SYBR	PLCz RA8	Unkn	RA8	71.00
H02	SYBR	β-actin RA8	Unkn		87.00

3.5 Sequencing

Figure 6. Sequencing data for PLC-zeta from *Rattus argentiventer* align with published sequence of PLC-zeta of *Rattus norvegicus*.

Figure 6 shows the sequencing data for PLCζ from *Rattus argentiventer* align with the sequence of PLCζ of *Rattus norvegicus*. The nucleotides coloured in red denote the difference between species showing 98% homology.

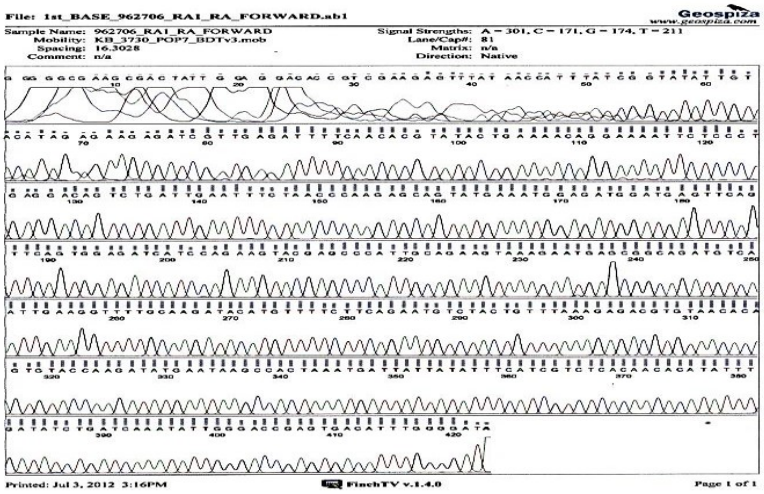


Figure 7:Chromatogram using Finch TV' software of PLC-zeta from Rattus argentiventer.

Figure 7 depict the chromatogram of PLCζ of *Rattus argentiventer* using the Finch TV software. Note the constant peaks confirming the sequence.

IV. Discussions

The results from the FA gel in fig. 1 showed two clear RNA bands observed from RNA samples of*Rattus argentiventer*. The two distinctive RNA bands were identified as 18S and 28S ribosomal RNA. The bands exhibited the integrity of the RNA samples used in the study. Living cells are classified into two groups namely prokaryotes and eukaryotes. Animals, plants and other cellular microbes such as fungi, protozoa and algae are categorised as eukaryotic cells whereas bacteria and cyanobacteria (blue-green algae) were prokaryotes. Eukaryotic cells have 80S ribosomes which are subsequently divided into small subunits (40S) and large subunits (60S). The small subunit contains 18S RNA (1900 nucleotides) and large subunits 28S RNA (4700 nucleotides), whereas prokaryotes consist of 70S ribosomes, containing 30S and 50S small and large subunits respectively. The small subunits in prokaryote has 16 S RNA (1540 nucleotides) and 23 S RNA (2900 nucleotides). S refers to Svedberg unit, which reflects the relative rate of sedimentation during ultrahigh speed centrifugation. The sample was taken from the testes of *Rattus argentiventer* which is a true eukaryote and therefore demonstrated the appearance of two distinctive bands. The preparation of RNA samplesis crucial in order to maintain the integrity of RNA. This is because RNA is easily degraded by RNAses which can be found elsewhere outside the study setting. The process of maintaining RNA integrity throughout the study is the first important step to ensure valid results. Theoretically the process of sample disruption in RNA samples preparation can be performed using either mechanical or chemical disruption (Sasidharan, et. al., 2012). The present study used mechanical disruption technique which included flash freezing such as grinding the samples in liquid nitrogen using mortar and pestle. Disruption includes the usage of lysis enzymes such as lyticase or zymolase which disrupts the cell wall of the tissue (Morozov et al., 2011).Once the samples were prepared, various commercial kits are available to extract RNA such as RNA Aqueous kit, Gen Elute mammalian total RNA kit, RNeasy mini kit and TRIzol LS reagent (Deng, et. al.,2005).However in this study, RNA Easy Blue reagent was used to extract the RNA samples from the rodent. The technique was able to extract RNA from the study samples because the principle of RNA extraction method was similar to other kits that have been previously reported. Several factors could lead to RNA degradation and Levy & Miller (1998) have reported that RNA degradation could result fromchanges in stability of temperature and pH of the storage of RNA samples. Therefore it is recommended to convert RNA to cDNA as soon as possible because of the RNA instability.

Figure 2shows the agarose gel electrophoresis of amplified products by conventional PCR and their molecular weights (420 bp). Gradient PCR was carried out at a range of annealing temperature from 50 to 60^{o}C. The annealing temperature which produced the strongest band intensity on the gel was selected. The result showed bands across lanes 1 to 10. There was an absence of bands in lane 11 and lane 12 while lane NTC acted as control. All efficiencies were automatically calculated by the software and readings appeared on the gel as

shown. The bands in lanes 5 to 10 indicated that the annealing temperature was optimized between 44.4 to 57.2^{o}C. The band in lane 7 which corresponded to temperature of 49.7^{o}C was chosen as the optimal annealing temperature. This temperature was appropriate and is agreeable with standard optimal annealing temperature for PCR process which falls between 40 to 60^{o}C (Roux, 2009).Fujimoto, et. al, (2004) had also reported a similar range of temperature which was optimal for the annealing process.

Figure 3 demonstrated successful RT-PCR amplification of PLCζcDNA from *Rattus argentiventer*.Clear bands of amplified products and β-actin as a positive control with their respective molecular weights were observed.The presence of PLCζ gene fragment was identified as dense bands across lanes 1 to 8. The primers used in the present study were able to amplify the cDNA. The size of the target gene fragment was expected to be between 400 to 500 bp while the size of β-actin (positive control) was expected to be between 300 to 400 bp. The results showed that the size of the target gene fragment and β-actin was 420bp and 370bp respectively. The result was comparable to the previous study by Fujimoto et. al., 2004, where single specific bands were observed at molecular weights of 420bp and β-actin as positive control at 370bp.The result also showed that no contamination occurred during the experiment as identified by the absence of band in the control lane (lane C) which acted as a negative control. β-actin was used as a positive control in the study because of its stability and consistency throughout all tissue samples(Katz et al., 2012). Similar positive control was used in a previous study of aquaporin and H+/K+-ATPase expression in transgenic mice (Carmosino et al., 2005). This study also confirmed the results of Jaffar, et. al., (2010) that PLCζ can be detected at the molecular weight of 420 bp

Figure 4 showed the result of qRT-PCR. This method was carried out according to the MIQE guidelines (Bustin et al., 2009), which states that qRT-PCR is required to be conducted in parallel with conventional PCR to reconfirm cDNA fragments (PLCζ) obtained. Amplification curves for RA1-RA8 denotes relative fluorescence unit (RFU) versus cycle of amplification. After 20 cycles of amplifications, threshold cycles, C_t were achieved, in which the fluorescence marker has been picked up by both β-actin and PLCζ. The difference between each cycle should be about 3.22 cycles while the result in the present study also showed approximately 3.22 cycles before the curves reached the plateau phase. In reference to the amplification curves, the amount of amplicon replication would double up in each cycle during the exponential phase. The process reaction would gradually decrease in the plateau phase because of enzyme limiting factor. In this study, PLCζ was detected in all samples whereby β-actin gene was designated as a positive control. Detection of PLCζ was further verified by the presence of β-actin in these samples. However the samples tested were not valid if there was no amplification of β-actingene. From the manual protocol of qRT-PCR, it was stated that the appropriate size of amplicon ranges from 75-200 bp in order to detect the presence of target gene fragment (Real-Time PCR Applications Guide Manual, 2006).Nevertheless, the present study utilized primers with higher molecular weights between 300 to 500 bp of product size and qRT-PCR was still able to identify the target gene fragments. This showed that the method was recommended because of its sensitivity, efficiency and less time consuming as compared to conventional PCR. In addition, it was reported that qRT-PCR was also used as a quantitative expression of PLCζ which required treated gene fragments (Aghajanpour et al., 2011). Other advantages of qRT-PCR include the production of quantitative data of melt curve and gene expression data in which conventional PCR would not be able to produce. However in the present study, the use of qRT-PCR was only to detect the target gene fragments and also to produce quantitative data of melt curve as gene expression studies were not performed.

Figure 5 shows melt peak for both PLCζ and β-actin.There was an absence of primer dimer curves as there was only one peak for each sample indicating that no contamination occurredduring the experiment. The melt data curve readings of qRT-PCR amplification of PLCζ from*Rattus argentiventer*were subsequently tabulated in Table 1. The results in Table 1 showed the consistency of melting temperature for both PLCζand β-actin. PLCζ melting temperature was between 79.5 to 81°C while β-actin melting temperature was between86.5 to 87°C. Melting temperature can be detected when the amplification curves reached the threshold baseline.

Figure.6 showed thepercentage of distribution of *Rattus argentiventer*PLCζ sequence data as compared with*Rattus norvegicus*PLCζ sequence data.The homology was confirmed using BLAST. The percentage of similarity was 98%. The difference of 2% was identified based on the position of the primers between Adenine (A), Guanine (G), Thymine (T) and Cytosine (C). The difference in position AGTC was highlighted in red.The strong homology showed that both *rattus* which came from the same genus have only 2% difference in the PLCζ sequence suggesting that only small changes occur as the rodentevolute from their ancestors. The sequence obtained could be further use to interfere with the expression of this protein by using interference RNA (siRNA). This method could also cause interference in other *rattus* too due to the small difference in the PLCζ sequence. The results of the gene sequence were also generated in the form of a chromatogram using Finch TV' software (Fig. 7) which served as a tool to generate sequence of gene fragments. BLAST from NCBI

in this study. The other tool to search sequences such as ClustalW from EMBL-EBI could also provide the alignment information.

V. Conclusions

This study was able to identify the presence of PLCζ enzyme in *Rattus argentiventer* using conventional PCR and further validated with qRT-PCR. The gene fragment of PLCζ enzyme was sequenced and the sequence was compared with standard PLCζ sequences using BLAST software. The homology of the PLCζ sequence with *Rattus norvegicus* was 98% suggesting only a small difference between species. The use of qRT-PCR could also assist in identification of PLCζ in samples in a more rapid, efficient and more sensitive approach. The findings of this study would give valuable baseline information for future researches to be carried out in the approach of identification, control and finally curbing the growth of the *Rattus argentiventer* population.

Acknowledgements

The research was made possible with the research grant from YayasanFelda and the donation of Rice Field Rats from MARDI.

References

[1.] Aghajanpour, S., Ghaedi, K., Salamian, A., Deemeh, M. R., Tavalaee, M., Moshtaghian, J., and Nasr-Esfahani, M. H. (2011). Quantitative expression of phospholipase C zeta, as an index to assess fertilization potential of a semen sample. Human Reproduction, 26(11), 2950-2956. doi: 10.1093/humrep/der285

[2.] Biorad Real-Time PCR Applications Guide Manual. Retrieved 23 August 2012 from http://www.genequantification.de/real-time-pcr-guide-bio-rad.pdf , 2006).

[3.] Buckle, A. P., Rowe, F. P., & Husin, A. R. (1984). Field trials of warfarin and brodifacoum wax block baits for the control of the rice field rat, rattus argentiventer in peninsular malaysia∗. Tropical Pest Management, 30(1), 51-58. doi: 10.1080/09670878409370851

[4.] Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M.,Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry, 55(4), 611-622. doi: 10.1373/clinchem.2008.112797

[5.] Carmosino, M., Mazzone, A., Laforenza, U., Gastaldi, G., Svelto, M., & Valenti, G. (2005). Altered expression of aquaporin 4 and H+/K+-ATPase in the stomachs of peptide YY (PYY) transgenic mice. Biology of the Cell, 97(9), 735-742. doi: 10.1042/BC20040138

[6.] Christensen, T., Lassen, P., & Elmeros, M. (2012). High Exposure Rates of Anticoagulant Rodenticides in Predatory Bird Species in Intensively Managed Landscapes in Denmark. Archives of Environmental Contamination and Toxicology, 63(3), 437-444.

[7.] Deng, M. Y., Wang, H., Ward, G. B., Beckham, T. R., & McKenna, T. S. (2005). Comparison of six RNA extraction methods for the detection of classical swine fever virus by real-time and conventional reverse Transcription–PCR. Journal of Veterinary Diagnostic Investigation, 17(6), 574-578. doi: 10.1177/104063870501700609

[8.] Fujimoto, S., Yoshida, N., Fukui, T., Amanai, M., Isobe, T., Itagaki, C. and Perry, A. C. F. (2004). Mammalian phospholipase Cζ induces oocyte activation from the sperm perinuclear matrix. Developmental Biology, 274(2), 370-383. doi: 10.1016/j.ydbio.2004.07.025

[9.] Jaffar, R.O., Noor Faezah, M.N., Cheah, Y.K.,Lee,L.H. and Sukardi, S. (2010). Identification of PLC-zeta in the testes of paddy rats (Rattus argentiventer). Malaysian Journal of Animal Science. 13 (1 & 2): 28 -33

[10.] Katz, Z. B., Wells, A. L., Park, H. Y., Wu, B., Shenoy, S. M., & Singer, R. H. (2012). β-Actin mRNA compartmentalization enhances focal adhesion stability and directs cell migration. Genes & Development, 26(17), 1885-1890. doi: 10.1101/gad.190413.112

[11.] Mizushima, S., Takagi, S., Ono, T., Atsumi, Y., Tsukada, A., Saito, N., & Shimada, K. (2009). Phospholipase C? mRNA expression and its potency during spermatogenesis for activation of quail oocyte as a sperm factor. Molecular Reproduction and Development, 76(12), 1200-1207. doi: 10.1002/mrd.21097

[12.] Morozov, I. A., Sachivkina, N. P., Kravtsov, E. G., Vasilyeva, E. A., Anokhina, I. V., Yashina, N. V., & Dalin, M. V. (2011). Damaging effects of lyticase on candida albicans and changes in the response of rat alveolar macrophages to the contact with yeast-like fungi. Bulletin of Experimental Biology and Medicine, 151(6), 705-708. doi: 10.1007/s10517-011-1421-x

[13.] Roux, K. H. (2009). Optimization and troubleshooting in PCR.Adapted from PCR Primer: A Laboratory Manual,Cold Spring Harbor Protocols.2nd edition. eds. .Dieffenbach and Dveksler). CSHL Press, Cold Spring Harbor, NY, USA. 2009(4), pdb.ip66. doi: 10.1101.

[14.] Sasidharan, K., Amariei, C., Tomita, M., & Murray, D. B. (2012). Rapid DNA, RNA and protein extraction protocols optimized for slow continuously growing yeast cultures. Yeast, 29(8), 311-322. doi: 10.1002/yea.2911

[15.] Saunders Christopher M, Larman Mark G, Parrington John, Cox Llewellyn J, Royse, J., Blayney, L. M., Lai, F. A. (2002). PLCζ: A sperm-specific trigger of Ca2+ oscillations in eggs and embryo development Development, 129(15), 3533-3544.

[16.] Swann, K., Larman, M. G., Saunders, C. M., & Lai, F. A. (2004). The cytosolic sperm factor that triggers Ca2+ oscillations and egg activation in mammals is a novel phospholipase C: PLCζ. Reproduction, 127(4), 431-439. doi: 10.1530/rep.1.00169

[17.] Tebes, S. J., & Kruk, P. A. (2005). The genesis of RNA interference, its potential clinical applications, and implications in gynecologic cancer. Gynecologic Oncology, 99(3), 736-741. doi: 10.1016/j.ygyno.2005.08.031

[18.] Wood, B. J., & Fee, C. G. (2003). A critical review of the development of rat control in malaysian agriculture since the 1960s. Crop Protection, 22(3), 445-461. doi: 10.1016/S0261-2194(02)00207-7

[19.] Yoneda, A., Kashima, M., Yoshida, S., Terada, K., Nakagawa, S., Sakamoto, A., and Watanabe, T. (2006). Molecular cloning, testicular postnatal expression, and oocyte-activating potential of porcine phospholipase Cζ. Reproduction, 132(3), 393-401. doi: 10.1530/rep.1.01018

[20.] Young, C., Grasa, P., Coward, K., Davis, L. C., & Parrington, J. (2009). Phospholipase C zeta undergoes dynamic changes in its pattern of localization in sperm during capacitation and the acrosome reaction. Fertility and Sterility, 91(5, Supplement), 2230-2242. doi: 10.1016/j.fertnstert.2008.05.021