UNIVERSITI PUTRA MALAYSIA

EVALUATION OF TOLL-LIKE RECEPTORS (TLRs) GENE EXPRESSION IN FELINE INFECTIOUS PERITONITIS VIRUS (FIPV)-INFECTED CELL LINE

NUR ATIQAH BINTI MUHAMMAD ALI

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NUR ATIQAH BINTI MUHAMMAD ALI

A project paper submitted to the
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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Evaluation of Toll-Like Receptors (TLRs) Gene Expression in Feline Infectious Peritonitis Virus (FIPV)-Infected Cell Line", by Nur Atiqah Binti Muhammad Ali and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 – Project

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DEDICATION

The love of my life, my parents

(Muhammad Ali Bin Kassim & Zaidah Binti Abdul Majid),

My siblings

(Saifullah and Amir Naim),

Lectures, Friends

&

DVM 2017

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	Percentage
CPE	Cytopathic Effect
CRFK	Crandell-Reese Feline Kidney
DCs	Dendritic cells
E	Envelope
FBS	Fetal Bovine Serum
FCoV	Feline Coronavirus
FECV	Feline Enteric Coronavirus
FIPV	Feline Infectious Peritonitis Virus
IFNs	Interferons
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-12	Interleukin-12
M	Membrane
MEM	Minimum Essential Media
ml	Millilitre
MOI	Multiplicity of Infection
nm	Nanometre
N	Nucleocapsid
NK	Natural Killer (NK)
ORFs	Open Reading Frames
PRRs	Pattern-Recognition Receptors

PAMPs	Pathogen Associated Molecular Patterns
ºC	Degree Celsius
qPCR	Real Time Polymerase Chain Reaction
RNA	Ribonucleic acid
S	Spike
$TCID_{50}$	Tissue Culture Infectivity Dose
TLRs	Toll-Like Receptors
TLR3	Toll-Like Receptor 3
TLR7	Toll-Like Receptor 7
TNF-α	Tumour Necrosis Factor -α
µg	Microgram
µl	Microliter

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek

Penilaian Ekspresi Gen Toll-Like Receptors (TLRs) Dalam Titisan Sel Yang Dijangkiti Feline Infectious Peritonitis Virus

Oleh

Nur Atiqah Binti Muhammad Ali

2017

Penyelia: Dr. Farina Mustaffa Kamal

Penyelia bersama: Dr. Tan Sheau Wei

Feline infectious peritonitis (FIP) ialah salah satu penyakit berjangkit utama yang membawa maut, melibatkan imuniti yang disebabkan oleh peritonitis berjangkit felin (FIPV), biotip paling virulen di dalam feline coronavirus (FCoV). Kajian terhadap komponen imun inat dalam penyakit FIP masih terhad. Pengenalan 'Toll-like receptors (TLRs)' adalah salah satu mekanisme yang digunakan dalam imuniti inat dalam mengesan corak molekular berulang di dalam patogen. Oleh itu, kajian ini dijalankan untuk mengesan modulasi ekspresi gen TLR3 dan TLR7 dalam titisan sel yang dijangkiti FIPV. Titisan sel Crandell-Reese Feline Kidney (CRFK) dijangkitkan dengan FIPV 79-1146 pada MOI = 0.01 dan ekspresi gen TLR3 dan TLR7 dikira

menggunakan ujian tindak balas berantai polimerase masa nyata (qPCR) pada titik masa yang berbeza (3 dan 24 jam selepas infeksi). Ekspresi untuk kedua-dua gen TLR3 dan TLR7 meningkat selepas 3 jam dan 24 jam apabila dibandingkan dengan kawalan. Tambahan pula, terdapat 5 kali ganda peningkatan nyata ekspresi gen TLR3 pada 24 jam selepas infeksi berbanding 3 jam selepas infeksi. Kuantiti virus pada titik masa yang berbeza tidak dikira, walaubagaimanapun, jangkitan virus telah disahkan menggunakan ujian PCR. Kesimpulannya, kedua-dua TLR3 dan TLR7 meningkat dalam jangkitan FIPV *in vitro*, tetapi korelasi antara ekspresi gen dan kinetik jangkitan FIPV memerlukan kajian lanjut.

Kata kunci: Peritonitis berjangkit felin virus, 'Toll-Like Receptors', Crandell-Reese Feline Kidney, ujian tindak balas berantai polimerase masa nyata

ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement for the course VPD 4999 – Project.

Evaluation of Toll-Like Receptors (TLRs) Gene Expression in Feline Infectious Peritonitis Virus (FIPV)–Infected Cell Line

By Nur Atiqah Binti Muhammad Ali

2017

Supervisor: Dr. Farina Mustaffa Kamal

Co-supervisor: Dr. Tan Sheau Wei

Feline infectious peritonitis (FIP) is one of the most infectious fatal diseases involving immune-mediated events caused by feline infectious peritonitis virus (FIPV), a highly virulent biotype of feline coronavirus. There are limited studies characterizing the innate immune component of FIP. Toll-like receptors (TLRs) recognition is one of the mechanisms used in innate immunity to detect repeated molecular pattern of pathogens. Thus, this study was conducted to evaluate the modulation of TLR3 and TLR7 genes expression in FIPV-infected cell line. Crandell-Reese Feline Kidney (CRFK) cell line was infected with FIPV 79-1146 at MOI = 0.01 and the expression of TLR3 and TLR7 were measured using Real-Time Polymerase Chain Reaction (qPCR) at different time points (3 and 24 hours post-infection (hpi)). For both TLR3 and TLR7 genes, the expression was upregulated at 3 and 24 hpi when

compared with control. In addition, there was a 5-fold significant increase of TLR3 gene expression at 24 hpi compared to 3 hpi. The viral load at the different time points was not measured; however, the infectivity of the virus was confirmed using conventional RT-PCR assay. In conclusion, it appears that both TLR3 and TLR7 were upregulated in FIPV infection *in vitro*, but the correlation between gene expression and kinetics of FIPV infection warrants further examination.

Keywords: Feline Infectious Peritonitis Virus, Toll-Like Receptors, Crandell-Reese Feline Kidney cell line, Real-Time Polymerase Chain Reaction

1.0 INTRODUCTION

Coronavirus is a virus in the family of *Coronaviridae* that has morphology of roughly spherical particle of approximately 60-200 nm in diameter, surface projections of about 20 nm long, and estimated molecular mass of 400 x 10^6. The name of 'coronavirus' is derived from the word "corona" which means crown referring to the characteristic appearance of the crown-like morphology virions observed under electron microscope.

There are 13 coronavirus species reported currently which infect multiple host species and one of them is known as Feline Coronavirus (FCoV) that causes infection in feline groups. Feline Infectious Peritonitis (FIPV) is one of the sub-groups under FCoV that have the ability to develop lethal disease known as Feline Infectious Peritonitis (FIP) especially in young cats. Recent evidence suggests that FIPV arises from the mutated version of avirulent, ubiquitous type of FCoV known as Feline Enteric Coronavirus (FECV).

Early detection of invaded microorganism by innate immunity is crucial in viral elimination. Innate immunity acts as a front line of host defense against invaded pathogen through germline encoded pattern-recognition receptor (PRRs) which includes Toll-Like Receptors (TLRs) and cytoplasmic receptors. These receptors are expressed by phagocytic cells including macrophages and dendritic cells. PRRs possess common characteristic such as recognize microbial components that are essential for microorganism survivability and therefore difficult for the microorganism to alter. Second, PRRs are expressed constitutively in the host and able to detect the pathogens at any stage of life cycle. Third, PRRs are germline encoded, nonclonal,

expressed on all cells of a given type, and independent of immunologic memory. Different PRRs react with specific pathogen associated molecular patterns (PAMPs), show distinct expression patterns and require specific signaling pathways leading to distinct immune responses.

There are several studies have been done on TLRs in coronavirus-associated disease, such as the study by Mazaleuskaya et al., (2012) which shown that TLR3-induced type I interferons (IFNs) provide protective roles in Murine Coronavirus infection. Besides, study done by Law et al., (2009) found that SARS-CoV did not stimulate the gene expression of TLRs in immature dendritic cells (DCs).

However, there is no study looking at TLRs gene expression in FIPV infection. Therefore, a justification for this project is due to the lack of study characterizing innate immunity in FIPV infection.

Thus, the objective for this study includes:

1. To measure TLR3 and TLR7 gene expression in FIPV-infected cell line at different time points

The hypothesis for this study was:

1. There is alteration of TLR gene expression in FIPV infection.

2.0 LITERATURE REVIEW

2.1 Feline Infectious Peritonitis

2.1.1 Feline Infectious Peritonitis Virus

Feline Infectious Peritonitis Virus (FIPV) is a virus of the family of *Coronaviridae* and *Alphacoronavirus* genus. It is known to be the largest enveloped virus about 60 - 200 nm in diameter containing single-stranded positive-sense RNA genome of approximately 29 kb in length. The viral RNA genome consists of 11 open reading frames (ORFs) which are the two major ORFs encode for replicase, four ORFs encode structural proteins (spike [S], membrane [M], nucleocapsid [N], and envelope [E] proteins) and another five ORFs encode non-structural proteins (3a, 3b, 3c, 7a, and 7b) (Hartmann, 2005). FIPV is one of the two biotypes of feline coronavirus (FCoV) in addition to Feline Enteric Coronavirus (FECV).

2.1.2 FCoV classification

FCoV biotype that causes a mild or inapparent infection in domestic cats is called FECV. On the other hand, FIPV is a highly virulent biotype of FCoV arises from mutation of FECV which could lead to fatal disease known as Feline Infectious Peritonitis (FIP). In addition, FECV are ubiquitous in the environment and present in mostly healthy cats population, whereas FIPV cause fatal, progressive, immunologically mediated disease which is characterized by severe systemic inflammatory damage of serosal membranes and extensive distribution of pyogranulomatous lesion in the vital organs including lungs, liver, lymph tissue and brain (Brown et al., 2009).

Based on virus neutralizing antibodies, both FECV and FIPV are further differentiated into serotype I and serotype II. Serotype I virus has a distinctively feline spike protein, while the spike protein of type II FCoV serotype is due to recombination of feline and canine enteric coronavirus (Pedersen, 2009). In addition, type I strain are more likely to cause clinical FIP in contrast to type II strain, which appeared to be more adaptable in tissue culture (Pedersen, 2009)

2.1.3 Immune component in FIPV infection

Immune component in FIPV infection comprised of innate immunity and adaptive immunity. For innate immunity, phagocytic cells such as natural killer (NK) cells are responsible for viral elimination during infection. Besides, cytokines possess a vital role in communication and interaction between cells, which may give protective or detrimental effect. Example of cytokines that give protecting role includes IL-10 which stimulates NK cells to positively regulates Fcγ I receptors and antibody-dependant cellular cytotoxicity that probably help in viral elimination (Myrrha et al., 2011). Other than that, IL-12 induces specific immunity, by mediating a positive feedback to amplify the Th1 response in cell-mediated immunity (Myrrha et al., 2011)

In contrast, cytokines that cause detrimental effect can be IL-1 and TNF-α. The phagocytic cells will strongly expressed these two cytokines during infection and allow the interaction with activated endothelial cells which would then release enzymes such as matrix metalloproteinase-9, which dissolve the vascular basement membranes at the sites of monocytes emigration (Hartmann, 2005). Besides, IL-6 contributes to the immune mediated destruction in central nervous system and lead to neurological clinical signs in FIP (Myrrha et al., 2011).

Acquired immunity on the other hand, consists of cell-mediated immunity (T-cell) and humoral mediated immunity (B-cell). It has been proposed that cat with weak cell-mediated immunity in combination with strong humoral immunity are likely to develop FIP as antibody in FIPV infection is not effective in viral elimination, and could enhance FIP development (Pedersen, 2014).

2.1.4 Immunopathogenesis of FIP

FIPV infect monocytes in the bloodstream and result in the release of cytokines such as IL-1 and TNF-α (Hartmann, 2005). These cytokines are causing upregulation of endothelial adhesin expression. Interaction between adhesion molecules and activated monocytes result in the release of metalloproteinase which cause loosen endothelial junction and allow diapedesis and leakage of plasma into extracellular space to occur.

The monocytes differentiated into activated macrophage which secretes pro-inflammatory cytokines and causing more endothelial adhesin to be expressed and attracting more monocytes and polymorphonuclear neutrophils and some lymphocytes to the area. These cells pack together to form a perivascular pyogranuloma (Pedersen, 2014). Cells in the pyogranuloma produce pro-inflammatory cytokines and chemokines, which attract more monocytes to the lesion, and release more metalloproteinases, disrupting junction between endothelial cells, and leading to leakage of plasma, resulting in effusion into the abdomen, thorax, or pericardium, which is one of the clinical signs in wet form FIP.

2.2 Toll-Like Receptors (TLRs)

Toll-Like Receptors (TLRs) is a type of the pattern-recognition receptors (PRRs) in innate immune system which help in early recognition of microbial component known as pathogen associated molecular patterns (PAMPs). The TLR family members are expressed on various immune and non-immune cells such as B-cells, NK (natural killer) cells, DCs, macrophages, fibroblast cells, epithelial cells and endothelial cells. However, TLRs are differentially localized within the cells as shown in Figure 1. TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the cell surface, whereas TLR3, TLR7, TLR8 and TLR9 are expressed in the endosomes.

The primary interest of this study is the TLR3 and TLR7. TLR3 is responsible in the recognition of double stranded RNA (dsRNA), which either present as part of virus genomic structure or generated during viral RNA replication (Takeda & Akira, 2005). TLR7 on the other hand, are important in the detection of single strand RNA (ssRNA) virus.

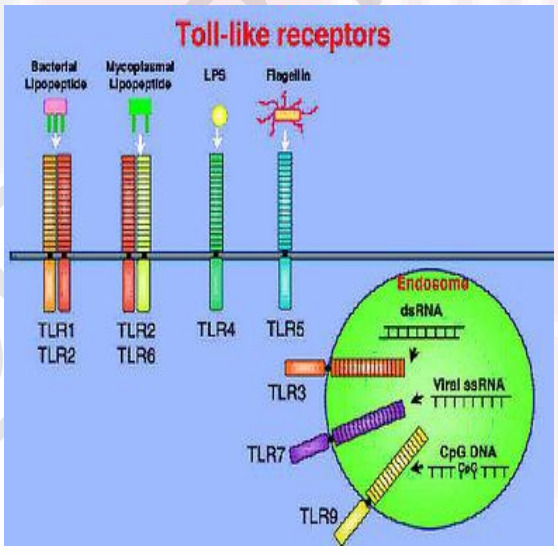


Figure 1: Location of TLRs within the cell

3.0 MATERIALS AND METHODS

3.1 Cell Subculturing

Crandell-Reese Feline Kidney (CRFK) was obtained from ATCC. Flasks that reached 90% confluency were subcultured. After discarding the old media, the flask was washed with 10 ml of 1X Phosphate Buffer Saline (PBS) twice. This procedure was performed in order to remove traces of the serum that would inactivate trypsin, thus will inhibit disassociation action of the trypsin. The wash solution was then discarded. Next, 2 ml of 0.5% trypsin-EDTA were added and incubated at room temperature for 1-2 mins until approximately 90% cells detached when observed under inverted microscope (Olympus, Japan). After that, 10 ml of pre-warmed minimal essential media (MEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin Streptomycin Glutamine (PSG) were added and pipetted up and down for several times to break the clumping cells and formed a homogenous mixture of cells and medium. The cells were then transferred into a T25 flask at concentration of $1x10^5$ cells/ml and incubated at 37°C, 5% CO_2.

3.2 Counting Cells Protocol

Cells were counted using Cell Counting Chamber Improved Neubauer Hemacytometer (Marien Field, Germany). Using 0.4% trypan blue stain, this procedure was performed to observe and count viable cells. Cells were washed with 1X PBS twice and 0.5% trypsin-EDTA was added to ensure at least 90% of cells were detached prior to cell counting. 1 ml of cells was taken and separated into an eppendorf tube.

Living cells can be seen as round and transparent under microscope observation while dead cells were edgy and blackened. The living cells in the large

chamber were counted and at least two large boxes were counted. By using this following formula, total number of cells can be obtained:

$$\frac{Total\ cell\ count}{No.of\ box\ counted}\ x\ dilution\ factor x\ 10^4 = \text{Total cell numbers/ml}$$

3.3 Virus Propagation

FIPV 79-1146 strain which obtained from ATCC was used in this study since this virus was well established strain and adaptable to cell culture. Once cells achieved 80-90% confluency, the cells were washed with 3 ml of 1X PBS. The cells were then inoculated with 1 ml of virus stock which has been thawed at room temperature.

The flask was incubated at 37°C with 5.0% CO_2 for one hour and was rotated every 10 mins. After the 1 hour incubation, 5 ml of media was added and the flask was incubated until cytopathic effect (CPE) was observed. Cells were observed every day and once 50% of cells died, the flask was subjected to freeze-thaw cycle for 3 times. Virus propagation can be done for several times in order to maintain the activation state of the virus and volume of virus stock.

3.4 TCID 50

Cell Seeding

The virus titer was calculated using Tissue Culture Infective Dose ($TCID_{50}$) method. CRFK cells were seeded onto a 12-well plate at concentration of $3x10^5$ cells/ml and incubated in CO_2 overnight.

Virus Inoculation

A 10-fold serial dilution of the virus stock was prepared. Briefly, 100 µl of virus stock was added in the first eppendorf tube and 900 µl media was added into each tube. Media were discarded from all wells. 100 µl of diluted virus was inserted

into each well according to the dilution factor except the first two well contained undiluted virus stock. Each dilution was subjected into two technical replicates. The plate was incubated at 37°C with 5.0% CO_2 and the plates were rotated every 10 mins for one hour. Lastly, 1 ml of MEM media was added into each well. CPE was observed every day and recorded at Day 4 and Day 5. Table which summarized the CPE result are attached in Appendix 2.

TCID calculation

The $TCID_{50}$/ml value was calculated using Spearman-Karber method and a virus dilution that gives an MOI=0.01 was calculated based on the $TCID_{50}$/ml value. This following formula was applied to obtain $TCID_{50}$ value:

TCID 50 value $= -1.0 - [(\frac{Total\ percentage\ of\ positive\ infected\ well}{100} - 0.5) \times (\log 10)]$

$= -1.0 - [(\frac{100+100+100+100+100+50}{100} - 0.5) \times (\log 10)]$

$= -6$

$= \mathbf{10^{-6}}$

10^{-6} $= \frac{1}{1\ 000\ 000}$

$= 0.000001$

0.000001 $= \frac{x}{1}$

X $= 1 \times 10^6$

$TCID_{50}$/ml $= 1 \times 10^6 \times 0.1$ ml

$= \mathbf{1 \times 10^5}$

Dilution factor $= \frac{0.7 \text{ x } 1 \text{ x } 10^5 \text{ x } 0.1}{0.01 \, x \, 300{,}000}$

= **2.33**

Volume of virus required = 15 wells x 100 µl

= 1500 µl

$= \frac{1500 \text{ µl}}{2.33}$

= **644 µl**

Volume of media to be added = 1500 µl – 644 µl

= **856 µl**

3.5 Virus Infection

A total of $3x10^5$ CRFK cells were seeded in two 6-well plate containing MEM supplemented with 10% FBS and 1% penicillin-streptomycin-glutamine (PSG). There were 2 different time points (t.p) – 3h, and 24h and an uninfected control. Each group had 3 different wells for biological replicates. The cells were incubated in 37°C, CO_2 incubator for 48h. Prior to virus inoculation, the media was aspirated completely and washed excessively using 1X PBS. The cells in 3h and 24h wells were infected with 100 ul of virus at an MOI of 0.01. Complete media was added into the uninfected wells. The plates were incubated in 37°C, CO_2 incubator for 1 hr to allow the virus to infect the cells. Excess media was aspirated completely and the cells were washed excessively using 1X PBS to remove any traces of non-adhered viral particles. New MEM media containing 1% FBS and 1% penicillin-streptomycin-glutamine (PSG) was added into the remaining wells and incubated in 37°C, CO_2 incubator.

3.6 RNA Extraction

Total RNA extraction was performed on the samples following manufacturer's recommendations (Machary-Nagel) as in Appendix 1 and stored in -60°C freezer. The total RNA was precipitated using 0.1 volume of 3M sodium acetate and 3 volume of 100% ethanol overnight in -70°C freezer to remove traces of salts and contaminants. The RNA was centrifuged at maximum speed for 30 mins before washing using 70% ethanol twice. The ethanol was then removed and the RNA was left to air-dry for 10 mins before it was re-dissolved in sterile deionized distilled water. The concentrated RNA was then stored in -70°C freezer.

3.7 Real Time PCR

Name	Forward	Reverse	Probe
TLR3	AAACTGTTGGA GGGTCTTGAG	ACGGCTATGGAA ACGTGCAAACC	GTCTTTCTCAC CTCCACATTCT
TLR7	GTCTCCCCTTT CTGTTTCTGT	GGCGGCAAGGAG AGGACATAAGG	CAGAGGAATCG TGCTTCTACTC
GAPDH	CATCAATGGAA AGCCCATCAC	CCCATTTGATGT TGGCGGGATCTC	CCCAGTAGACT CCACAACATAC

Table 1: TLR-specific primers and probes sequence for real-time PCR

1 – 2 µg of total RNA was used to synthesize cDNA with a final volume of 20 µl. The reactions for real time PCR were performed using SensiFAST^{TM} SYBR No-ROX One Step kit (Bioline Ltd, UK). 2 µl of cDNA is then mixed with 1X Sensifast Probe No-Rox Mix, 400nM of forward and reverse primer for TLR3 or TLR7 or GAPDH (Ignacio et al., 2005), 100nM of probe for TLR3 or TLR7 or GAPDH (Ignacio et al., 2005), and sterile deionized distilled water to a final reaction volume of 20 µl (BioLine). The reaction was done in triplicates for each biological replicate. The reactions were run on Bio-Rad CFX96^{TM} Real-Time PCR, with C1000^{TM} Thermal Cycler (Bio-Rad Laboraties, USA) with a heat cycle protocol of 95ºC for 2 mins 1

cycle, 40 cycles of 95ºC for 15s, 55ºC (TLR3) or 54ºC (TLR7 & GAPDH) for 15s, and 72ºC for 10s.

3.8 Conventional PCR

Name	Forward	Reverse	Probe
7b	GATTTGATTTGGC AATGCTAGATTTA	TCCGCTATGACGA GCCAACAATGGA	AGCTAACGTCTGG ATCTAGTGATTGTT

Table 2: FIPV 79-1146 (7b) specific primer and probe in conventional PCR assay

Infectivity of virus was confirmed with conventional PCR assay. The reactions were made by mixing 2 µl of cDNA with 2X Sensifast Probe No-Rox Mix, 10 µM of forward and reverse primer for 7b gene (Gut el al., 1999) , 10 µM of probe, and sterile deionized distilled water to reach final reaction volume of 50 µl (BioLine). The reactions were run on a thermal cycler (C1000 Touch™ Thermal Cycler, California) and the PCR reaction conditions were as follows; one cycle at 95ºC for 1 mins, 35 cycles of 95ºC for 15s, 52ºC for 15s, and 72ºC for 10s. Agarose gel electrophoresis was then run to visualize the DNA fragment by using 2% Agarose gel and Bio-Rad PowerPac 300 (USA). The presence of band was visualized using Bio-Rad Gel Doc™ (USA).

3.9 Statistical Analysis

GraphPad Prism® software (GraphPad Software Inc, USA) was used to perform statistical analysis. The comparison of TLR3 and TLR7 gene expression at different time points was analysed using one-way ANOVA with P-value < 0.05 was considered significance.

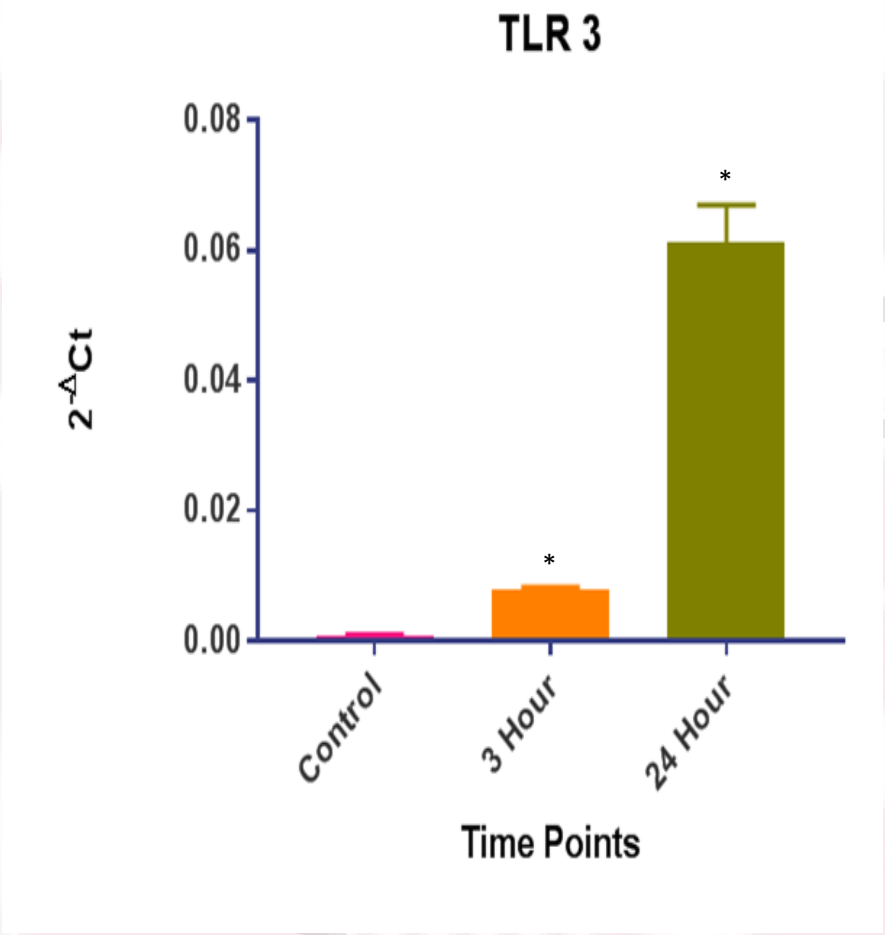
4.0 RESULTS & DISCUSSION

Quantification of TLR 3 and TLR 7 mRNA were done by normalizing the threshold value (Ct value) of the target gene with the threshold value (Ct value) of the reference gene, GAPDH, as in Appendix 3 by using this formula.

Tested Gene Ct Value – Reference Gene Ct Value (GAPDH) =ΔCt Value

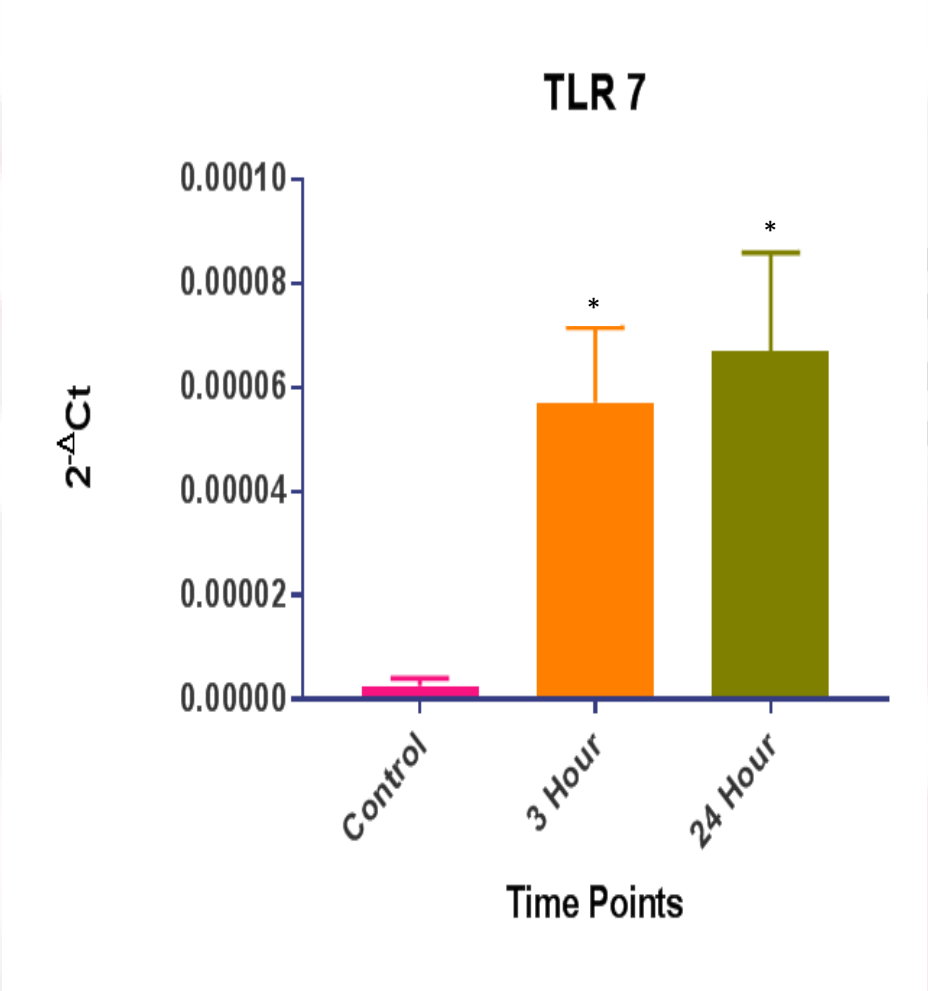
The n-fold difference relative to GAPDH was then calculated using $2^{-\Delta Ct}$ (Kipar et al., 2001) with the assumption that the amplification efficiency was at 2. Mean for each time points of TLR3 and TLR7 gene expression were plotted and considered significant if the P-value < 0.05.

Figure 2: TLR3 gene expression at different time points



Significant upregulation of TLR3 gene expression at different time points was observed when compared to control ($P<0.0001$) and Tukey's multiple comparison test revealed that there is a significant difference between control to 3- hour post-infection (hpi) ($P = 0.0057$), control to 24 hpi ($P <0.0001$), and between 3 and 24 hpi ($P <0.0001$) as shown in Figure 1.

Figure 3: TLR7 gene expression at different time points



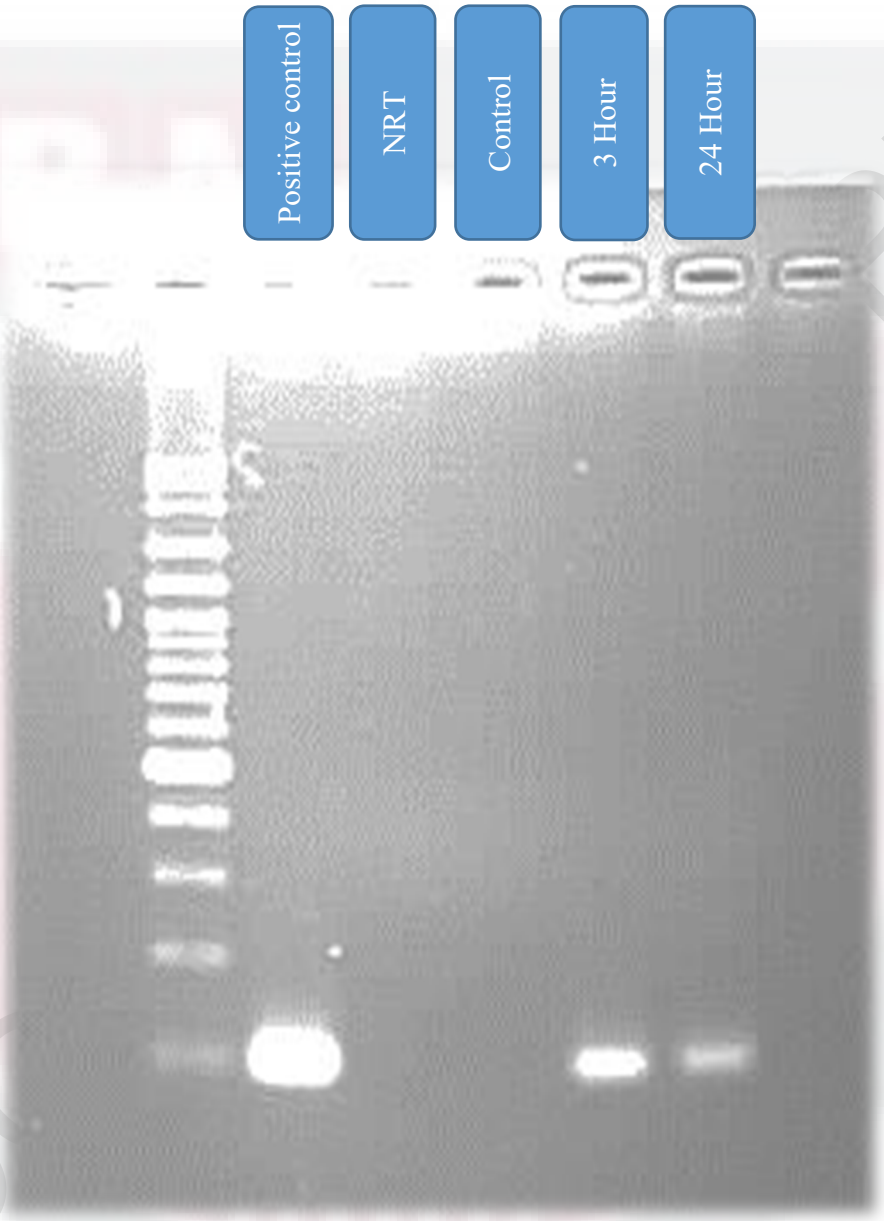
Similiarly, there is significant upregulation of TLR7 gene expression at different time points when compared to control ($P<0.0001$) and Tukey's multiple comparison test revealed that there is significance difference between control to 3 hpi ($P=0.0057$) and control to 24 hpi ($P<0.0001$). However, no significance difference of TLR7 gene expression was observed between 3 and 24 hpi ($P<0.0001$) as shown in Figure 2.

Upregulation of TLR3 and TLR7 genes expression started to occur at 3 hpi and markedly elevated at 24 hpi particularly the TLR3 expression with a 5-fold increase when compared to control. This is in agreement with a study conducted by Dewerchin

et al. (2005) which observed appearance of viral antigen in positive-infected cells as early as between 3 to 6 hpi. In addition, there was a significant increase in infected cells between 12 to 24 hpi. In fact, 86% of the cells may show cytoplasmic expression of viral antigen and 75% surface expression at 24 hpi.

Furthermore, the TLR7 gene expression is much lower compared to TLR3 gene expression in CRFK-infected cell line. This result is consistent with study conducted by Ignacio et al., (2005) which revealed that TLR7 is underexpressed by CRFK cells compared to TLR3. In addition, TLR 3 and TLR7 were found to be highly expressed in feline T-cell line such as MCH5-4 and Mya-1, respectively.

Figure 4: Virus infectivity confirmation



Virus infectivity was confirmed with conventional PCR where presence of virus was detected at 3 and 24 hpi compared to control (Figure 3). This indicates the upregulation of TLR3 and TLR7 gene expressions were due to the FIPV infection. The intensity of the band was reduced at 24 hpi compared to 3 hpi which may indicate reduce viral replication at 24 hpi which is in line with the study by Law et al., (2005)

which observed that replication of SARS-CoV virus peaked at 3 hpi but started to decline at 9 to 24 hpi.

Stimulation of TLR3 and TLR7 will result in the production of pro-inflammatory cytokines and type I interferons (IFNs) via its cytoplasmic Toll/IL/1 receptor (TIR) domain (Xagorari & Chlichlia, 2008). In addition, TLR3 appears to activate a downstream protein known as TIR-containing adaptor inducing IFN-beta (TRIF/TICAM-1). This adaptor protein is responsible for the induction of IFN-alpha and IFN-beta gene expression by TLR3 (Kumar et al., 2009). On the other hand, TLR7 activates the TIR-containing cytosolic adapter protein, MyD88 pathway which is also responsible in type I IFNs production. Production of Type I IFNs regulates the activity of innate cells such as macrophages, NK cells, and antigen-presenting cells (pDC) (Hertzog, 2012). These interactions and via the production of IFNs and other chemokines or cytokines could shape the development of adaptive response.

Besides, the roles of Type I IFNs are to induce resistance to viral replication by sending signals in autocrine and paracrine manners. This will in turn stimulate an anti-viral state in surrounding cells by inducing transcription of genes involved in apoptosis, anti-growth as well as innate and adaptive immune cell activations (Hertzog, 2012). Furthermore, innate cells directly communicate with adaptive immune response cells by upregulating their surface receptors and co-stimulatory molecules.For example, differentiation of T cell into effector and memory cells is regulated by the type I IFNs. Therefore, the overall effects of type I IFNs appear to be crucial for a productive T-cell response, especially in viral infections.

Type I IFNs helps in regulation of B cells, which plays an important role in humoral immunity. Effect of IFNs on B-cell response towards viral infection can be both direct and indirect, which results in the production of cytotoxic and neutralizing antibodies, surface proteins and secretion of cytokines by the B-cells.

5.0 CONCLUSIONS

In conclusions, this study revealed that there is significant upregulation of TLR3 and TLR7 genes expression in FIPV-infected cell line at different time points. However, the correlation between the quantity of virus and gene expression could not be determined.

6.0 RECOMMENDATIONS

It is recommended that generation of standard curve should be incorporated incorporated which is particularly important for underexpressed gene. In addition, virus infection using higher MOI need to be evaluated to determine whether it has any effects on the TLRs gene expression.

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APPENDIX

APPENDIX 1

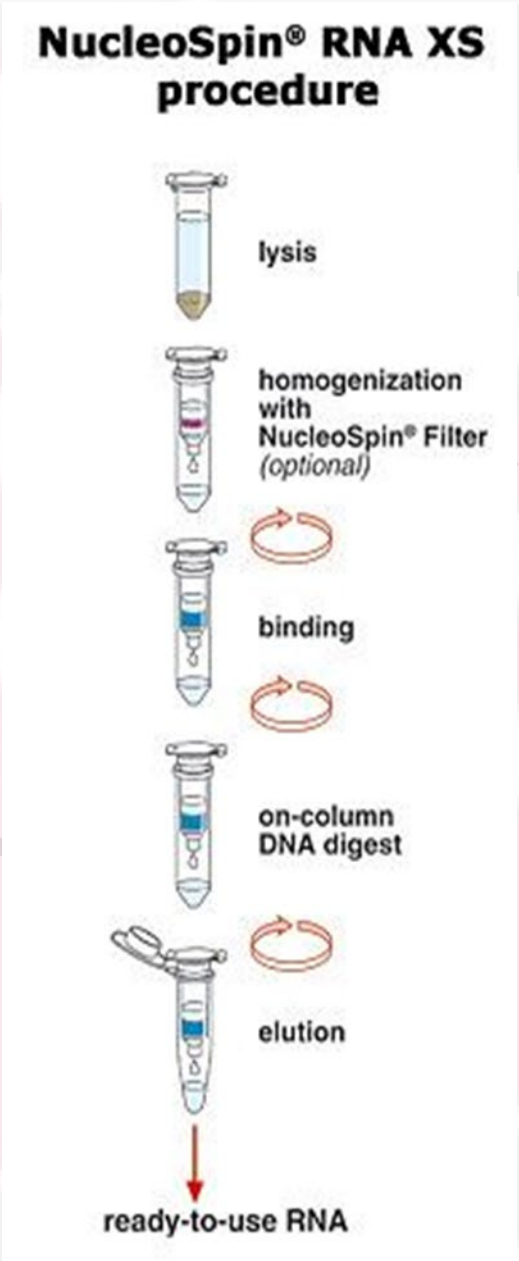
Date: 31/01/2017 (Day 4)

10^{-1}	+ve	**10^{-3}**	+ve	**10^{-5}**	+ve	**10^{-7}**	-ve	**10^{-9}**	-ve	**C**	-ve
10^{-1}	+ve	**10^{-3}**	+ve	**10^{-5}**	+ve	**10^{-7}**	-ve	**10^{-9}**	-ve	**C**	-ve
10^{-2}	+ve	**10^{-4}**	+ve	**10^{-6}**	+ve	**10^{-8}**	-ve	**10^{-10}**	-ve	**C**	-ve
10^{-2}	+ve	**10^{-4}**	+ve	**10^{-6}**	-ve	**10^{-8}**	-ve	**10^{-10}**	-ve	**C**	-ve

Date: 01/02/2017 (Day 5)

10^{-1}	+ve	**10^{-3}**	+ve	**10^{-5}**	+ve	**10^{-7}**	-ve	**10^{-9}**	-ve	**C**	-ve
10^{-1}	+ve	**10^{-3}**	+ve	**10^{-5}**	+ve	**10^{-7}**	-ve	**10^{-9}**	-ve	**C**	-ve
10^{-2}	+ve	**10^{-4}**	+ve	**10^{-6}**	+ve	**10^{-8}**	-ve	**10^{-10}**	-ve	**C**	-ve
10^{-2}	+ve	**10^{-4}**	+ve	**10^{-6}**	-ve	**10^{-8}**	-ve	**10^{-10}**	-ve	**C**	-ve

APPENDIX 2


NucleoSpin® RNA XS procedure
lysis
homogenization with NucleoSpin® Filter (optional)
binding
on-column DNA digest
elution
ready-to-use RNA

APPENDIX 3

TLR 3		Control			3 Hr			24 Hr		
		Experimental Well 1	Experimental Well 2	Experimental Well 3	Experimental Well 1	Experimental Well 2	Experimental Well 3	Experimental Well 1	Experimental Well 2	Experimental Well 3
2^-ΔCt Value	Replicate 1	0.000917503	0.000553166	0.022405551	0.008431471	0.007921558	0.055552668	0.063813258	0.031906629	0.054033577
	Replicate 2	0.000838443	0.001145347	0.022718321	0.007188966	0.0078125	0.059128603	0.059954007	0.033725882	0.055939067
	Replicate 3	0.000826900	0.000911165	0.016862941	0.008144264	0.007921558	0.042101049	0.06515411	0.040666933	0.06886907

TLR7		Control			3 Hr			24 Hr		
		Experimental Well 1	Experimental Well 2	Experimental Well 3	Experimental Well 1	Experimental Well 2	Experimental Well 3	Experimental Well 1	Experimental Well 2	Experimental Well 3
2^-ΔCt Value	Replicate 1	4.14556E-06	3.92194E-06	0.00012207	2.15792E-05	6.31876E-05	7.72557E-05	4.53041E-05	0.000155586	7.15842E-05
	Replicate 2	1.23248E-06	1.67199E-06	0.000129031	1.59068E-05	5.0268E-05	5.61638E-05	4.9576E-05	0.000183746	0.000107752
	Replicate 3	3.89485E-06	1.5818E-06	0.000212537	2.5134E-05	3.45729E-05	6.18872E-05	9.77869E-05	6.31876E-05	7.41086E-05