UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF TAQMAN-BASED REAL-TIME RT-PCR ASSAY FOR QUANTITATIVE DETECTION OF FELINE MORBILLIVIRUS (FEMV) AND PHYLOGENETIC ANALYSIS OF MALAYSIAN FEMV ISOLATES

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FPV 2021 2

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

April 2021

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

DEVELOPMENT OF TAQMAN-BASED REAL-TIME RT-PCR ASSAY FOR QUANTITATIVE DETECTION OF FELINE MORBILLIVIRUS (FEMV) AND PHYLOGENETIC ANALYSIS OF MALAYSIAN FEMV ISOLATES

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Feline morbillivirus (FeMV) of genus Morbillivirus is a novel emerging virus of domestic cats, linked with the development of CKD. Several quantitative diagnostic assays have been developed for the detection of FeMV such as reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR); however, none of the published assays targets the *N* gene of FeMV. *N* gene is one of the most conserved genes in morbilliviruses which also plays an important role in RNA synthesis by RdRp complex. In view for a specific and sensitive assay targeting local isolates, the objectives of this study were to develop Taqman-based qRT-PCR assay for the quantitative measurement of FeMV based on the sequence of *N* gene of local isolates and to assess the sensitivity and specificity of the developed qPCR assay in detecting FeMV. Sequence analyses of FeMV-Malaysia isolates were performed to develop specific primers targeting *N* gene. Phylogenetic analysis shown that the local isolates were closely related to the isolates from China, Thailand and Japan. Subsequently, a set of primers was designed and used in qRT-PCR assay which demonstrated a high specificity with no amplification signal towards other morbilliviruses and other feline viruses. Lowest limit of detection for the developed assay was at 1.74 x 10^{-4} copies/μL. The CV values for inter- and intra-assay variation were low, ranging from 1.38% - 2.03%, and 0.34% - 0.53%, respectively. Besides, the developed qRT-PCR assay was tested using cats' urine and kidney samples. The findings were then compared with the detections using conventional RT-PCR. The developed qRT-PCR assay detected FeMV in 35.2% of cats' samples compared to 15.5% by conventional RT-PCR. In conclusion, the developed assay of qRT-PCR has a high specificity and sensitivity in detecting FeMV compared to conventional RT-PCR, thus can be utilized as diagnostic tools and to determine possible association with CKD occurrence in cats.

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

PENGHASILAN UJIAN MASA NYATA RT-PCR BERASASKAN TAQMAN UNTUK PENGESANAN KUANTITATIF MORBILLIVIRUS FELIN (FEMV) DAN ANALISIS FILOGENETIK FEMV ISOLAT MALAYSIA

Oleh

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Feline morbillivirus (FeMV) daripada genus Morbillivirus ialah virus baru muncul bagi kucing domestik, dikaitkan dengan perkembangan CKD. Beberapa ujian diagnostik kuantitatif telah dibangunkan untuk pengesanan FeMV seperti amplifikasi gelung dimediasi isotermal transkriptas membalik (RT-LAMP) dan reaksi masa nyata rantai polimerase transkriptas membalik (qRT-PCR); walau bagaimanapun, tiada ujian yang diterbitkan menyasarkan gen *N* FeMV. Gen *N* adalah salah satu gen yang paling terpelihara dalam morbillivirus yang juga memainkan peranan penting dalam sintesis RNA oleh kompleks RdRp. Memandang kepada pengujian yang khusus, ujian khusus dan sensitif yang menyasarkan pencilan tempatan, objektif kajian ini adalah untuk membangunkan ujian qRT-PCR berasaskan Taqman untuk pengukuran kuantitatif FeMV berdasarkan urutan gen N pencilan tempatan dan untuk menilai sensitiviti dan kekhususan. daripada ujian qPCR yang dibangunkan dalam mengesan FeMV. Analisis jujukan bagi isolat FeMV-Malaysia telah dilakukan untuk membangunkan primer khusus yang menyasarkan gen N. Analisis filogenetik menunjukkan bahawa isolat tempatan berkait rapat dengan isolat dari China, Thailand dan Jepun. Berikutan itu, satu set primer telah direka dan digunakan dalam ujian qRT-PCR yang menunjukkan kekhususan tinggi tanpa isyarat amplifikasi terhadap morbillivirus lain dan virus kucing lain. Had pengesanan terendah dari ujian yang telah dihasilkan adalah 1.74 x 10-4 salinan/µL. Nilai CV untuk kebolehubahan ujian antara dan intra ujian adalah rendah, masing-masing antara julat 1.38% - 2.03%, dan 0.34% - 0.53%. Selain itu, ujian qRT-PCR yang dihasilkan juga diuji menggunakan sampel urin dan ginjal dari kucing. Penemuan menggunakan qRT-PCR kemudian dibandingkan dengan pengesanan daripada RT-PCR konvensional. Ujian qRT-PCR yang dihasilkan berjaya mengesan FeMV

dalam 35.2% sampel kucing berbanding 15.5% yang telah dikesan oleh RT-PCR konvensional. Kesimpulannya, ujian qRT-PCR yang dihasilkan adalah lebih spesifik mempunyai kekhususan dan sensitif untuk mengesan FeMV berbanding dengan RT-PCR konvensional, maka ia boleh digunakan sebagai alat diagnostik untuk merancang rawatan awal untuk CKD pada kucing.

ACKNOWLEDGEMENTS

All praise to Almighty Allah, with His blessing for granted me the chance and courage for me to go through my postgraduate study.

Firstly, I would like to thank Dr Farina Mustaffa Kamal, as my supervisor for her support, patience and motivation throughout my study. Furthermore, I also would like to express my gratitude towards my supervisory committee members, Prof Abdul Rahman Omar and Dr Tan Sheau Wei for their guidance and meaningful advice whether on life advice or in my research-related issue. I am also greatly appreciated towards participating private clinics and animal shelter centres also UVH staffs especially Dr Hemadevy Manoraj and Dr Khor Kuan Hua for their kind assistance in collecting the biological samples.

Next, to my previous and current lab team members, Muhammad Azlil, Megat Hamzah, Wallace Chee, Nurul Najwa Ainaa, Nur Afiqah and Lou Chan Hui. Thank you for your time and helps throughout my study. To all the staff and students of Virology Laboratory of Faculty of Veterinary Medicine, especially Pn Siti Khatijah Muhammad, Pn Wan Nur Ayuni Wan Noor, Mr Azman Asmat, Mr Rusdam Awang, Krishnan, Kiven, Jamilu, Natasha and Sifa. I also would like to thank Kak Farah, Mei Ho and Kak Suraya for their unconditional kind assist and knowledge.

I also would like to thank my friends; Siti Hajar, Nur Nadhirah, Nur Ain Najwa, Nadia, Nurul Ashiqin, Siti Nor Azizah and Nur Syazana that who have been with me through thick and thin. Thank you for always stay with me. My deepest gratitude towards my parents, Mr Makhtar Bin Mohamed and Mrs Kamaliah Binti Mohd Noor, also my siblings for their endless supports and motivations. Finally, I would like to thank everyone who has been contributed directly or indirectly throughout my study.

Siti Tasnim Binti Makhtar

This thesis was submitted to the 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

%	Percentage
℃	Degree celcius
∞	Infinity
~	Approximate
μg	Micro gram
μL	Micro litre
μm	Micro metre
amp/kan	Ampicillin/kanamycin
ATU	Additional transcription unit
BLAST	Basic Local Alignment Search Tool
BLD	Below limit of detection
bp	Base pair
CCoV	Canine coronavirus
cDNA	Complementary deoxyribonucleic acid
CDV	Canine distemper virus
CeMV	Cetacean morbillivirus
CKD	Chronic kidney disease
CO_2	Carbon dioxide
CPE	Cytopathic effect
CPIV	Canine parainfluenza virus
CPV	Canine parvovirus
Cq	Quantitation cycle
CRFK	Crandell-Reese feline kidney
cRNA	Complementary ribonucleic acid

CV	Coefficient variations
DMV	Dolphin morbillivirus
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
F	Fusion
F_0	Inactive precursor *F* protein
FCoV	Feline coronavirus
FeLV	Feline leukemia virus
FeMV	Feline morbillivirus
FeMV-GT1	Feline morbillivirus genotype 1
FeMV-GT2	Feline morbillivirus genotype 2
FLUTD	Feline lower urinary tract disease
FURD	Feline upper respiratory disease
FMDV	Foot-and-mouth-disease virus
G	Glycoprotein
G	Guanine
g	gram
H	Haemagglutinin
HBV	Hepatitis B virus
HK	Hong Kong
HN	Haemagglutinin-neuraminidase
hr	Hour
IACUC	Institutional Animal Care and Use Committee
IDT	Integrated DNA Technologies
IFA	Immunofluorescence assay

IgG	Immunoglobulin G
IHC	Immunohistochemistry
kb	Kilobases
kDA	Kilodalton
L	Large protein
LAMP	Loop-mediated isothermal amplification
M	Matrix
M	Molar
MEGA	Molecular Evolution Genetic Analysis
MeV	Measles virus
min	Minute
mL	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
N	Nucleocapsid
n	Number
N/A	Not available
NCBI	National Center for Biotechnology Information
NDV	Newcastle disease virus
ng	Nano gram
NLS	Nuclear localization signal
nm	Nano metre
nt	Nucleotide
NTC	No template control
OIE	Office International des Epizooties

ORF	Open reading frame
P	Phosphoprotein
PBS	Phosphate-buffer saline
PCR	Polymerase chain reaction
PDV	Phocine distemper virus
PMV	Porpoise morbillivirus
PPRV	Peste des petits ruminant virus
PPR	Peste des petits ruminant
PVRL4	Poliovirus-receptor-like-4
PWMV	Pilot-whale morbillivirus
qRT-PCR	Real-time reverse transcription polymerase chain reaction
R^2	Coefficient of determination
RBP	Receptor-binding protein
RdRp	RNA-directed RNA polymerase
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RP	Rinderpest
RPA	Recombinase polymerase amplification
RPV	Rinderpest virus
RT-LAMP	Reverse transcription loop mediated isothermal amplification
RT-PCR	Reverse transcription polymerase chain reaction
RT-RPA	Reverse transcription recombinase polymerase amplification
SAMRS	Self-avoiding Molecular Recognition System
SD	Standard deviation
SH	Small hydrophobic

SLAM	Signaling lymphatic activation molecule
SOC	Super optimal broth
SSB	Single stranded DNA binding protein
$TCID_{50}$	Median tissue culture infectious dose
TIN	Tubulointerstitial nephritis
tM	Transmembrane protein
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
UVH	University Veterinary Hospital

CHAPTER 1

INTRODUCTION

RNA genome of feline morbillivirus (FeMV) is negative-sense, non-segmented and single stranded characterized under family *Paramyxoviridae*, genus *Morbillivirus* (Amarasinghe et al., 2019). Members of morbilliviruses are known to infect both terrestrial and marine animals and are highly contagious (Pfeffermann et al., 2018). An infection of morbilliviruses can cause mild to critical gastrointestinal and respiratory diseases with acute immunosuppression towards the host which render them susceptible to opportunistic infections resulting in life-threatening complications such as pneumonia.

First detection of FeMV was in Hong Kong in 2012. Then, FeMV detection has been monitored in several countries for example United States, Germany, Japan, Brazil, Italy, Thailand, Turkey and Malaysia (Woo et al., 2012; Furuya et al., 2014; Lorusso et al., 2015; Claire et al., 2016; Chaiyasak and Techangamsuwan, 2017; Darold et al., 2017; Yilmaz et al., 2017; Mohd Isa et al., 2019). Based on the sequence and phylogenetic analyses of FeMV, it was classified as a novel virus due to its lower than 80% of nucleotide similarities to previously known paramyxovirus (Woo et al., 2012). However, in term of gene organization, it contains six genes (3'-*N*-*P*/*V*/*C*-*M*-*F*-*H*-*L*-5') which is similar to other morbilliviruses. FeMV detection study in Malaysia was conducted in 2015 of which 39.4% cats were found positive with highest detection in kidney (80%) (Mohd Isa et al., 2019).

Several initial studies of FeMV have linked chronic kidney disease (CKD) with FeMV infection (Woo et al., 2012; Furuya et al., 2014; Sutummaporn et al., 2019). This has raised a concern among veterinarians and cats' owner as CKD has been recorded as one of the most typical cause lead to death in domestic cats among age older than five years (O'Neill et al., 2015; Cannon, 2016). Following that, more researches have been performed to investigate the relationship between CKD and FeMV; however, a distinct association has yet been convincing (McCallum et al., 2018). In addition, canine distemper virus (CDV), another member of morbilliviruses has been proved possess the ability to be transmitted across different species (Martella et al., 2008). Besides, zoonotic concern has been raised regarding this FeMV infection in domestic cats as cats are popular as companion animal, but this concern can be eliminated as a study on FeMV-cell line infection has proved that FeMV showed no infection towards human cell lines (Sakaguchi et al., 2015).

In addition, several molecular investigations have been performed using conventional RT-PCR detecting *L* gene such as study done by Woo et al. (2012), Furuya et al. (2014) and Stranieri et al. (2019). Even though conventional RT-PCR is relatively cheaper; however, it is laborious and less sensitive compared to quantitative real-time RT-PCR (qRT-PCR). There are also qRT-PCR assays that

have been developed based on *L* gene and *P/V/C* gene but none of these studies target for nucleocapsid protein (*N* gene) of FeMV (Furuya et al., 2015; De Luca et al., 2018). *N* gene of FeMV was opted to become a target gene in this study as the *N* gene of morbillivirus is a well-conserved gene and major viral protein which folds and protects the viral RNA (Diallo, 1990).

Hence, the objectives of this study are:

1. To sequence and conduct phylogenetic analysis of the nucleocapsid (*N*) gene feline morbillivirus (FeMV) of Malaysia isolates.

2. To develop the Taqman-based real-time reverse transcription polymerase chain reaction (qRT-PCR) assay for the quantitative measurement of FeMV based on the *N* gene of Malaysia isolates.

3. To assess the specificity and sensitivity of the developed qRT-PCR targeting *N* gene in detection of FeMV from cats' urine and kidney samples.

Hypotheses of the study:

1. **H_0:** There is no significant difference in clustering FeMV-*N* gene Malaysia isolates with other outgroups from species of genus Morbillivirus
   H_1: There is significant difference in clustering FeMV-*N* gene Malaysia isolates with other outgroups from species of genus Morbillivirus

2. **H_0**: There is no significant difference in the performance of qRT-PCR compared to conventional RT-PCR in detection of FeMV-*N* gene
   H_1: There is significant difference in the performance of qRT-PCR compared to conventional RT-PCR in detection of FeMV-*N* gene

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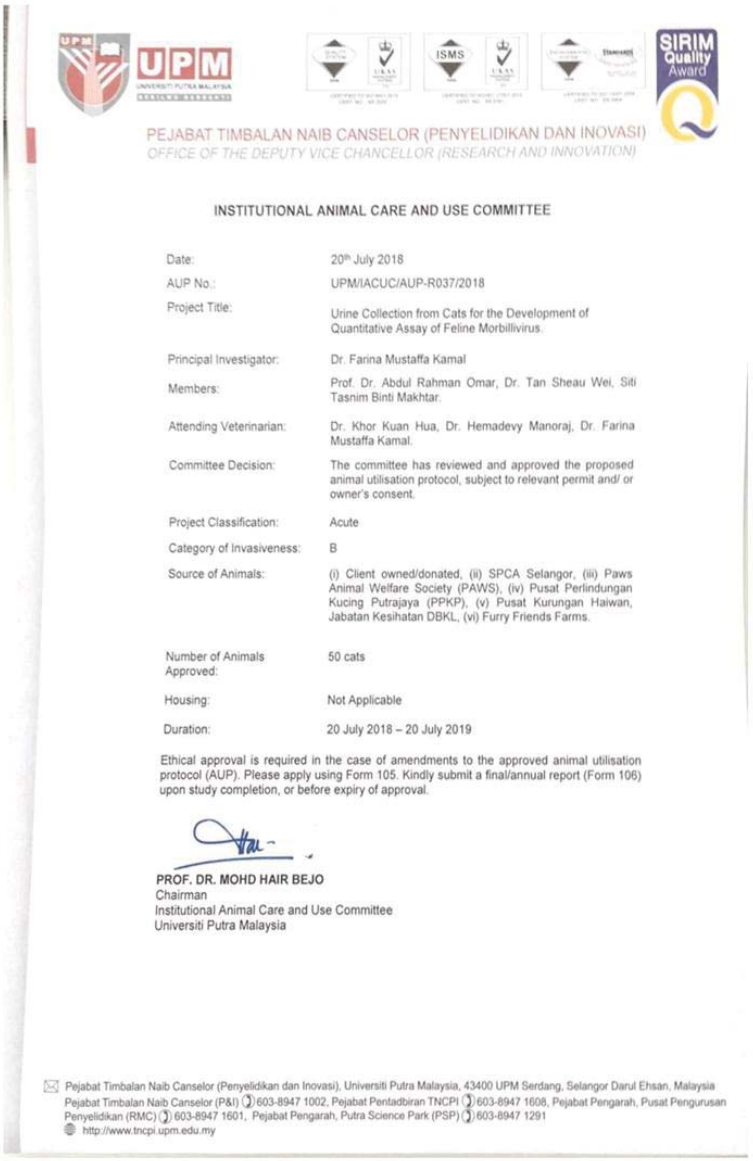
APPENDICES

Appendix 1

IACUC Approval Letter



PEJABAT TIMBALAN NAIB CANSELOR (PENYELIDIKAN DAN INOVASI)
OFFICE OF THE DEPUTY VICE CHANCELLOR (RESEARCH AND INNOVATION)

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Date:	20th July 2018
AUP No.:	UPM/IACUC/AUP-R037/2018
Project Title:	Urine Collection from Cats for the Development of Quantitative Assay of Feline Morbillivirus.
Principal Investigator:	Dr. Farina Mustaffa Kamal
Members:	Prof. Dr. Abdul Rahman Omar, Dr. Tan Sheau Wei, Siti Tasnim Binti Makhtar.
Attending Veterinarian:	Dr. Khor Kuan Hua, Dr. Hemadevy Manoraj, Dr. Farina Mustaffa Kamal.
Committee Decision:	The committee has reviewed and approved the proposed animal utilisation protocol, subject to relevant permit and/ or owner's consent.
Project Classification:	Acute
Category of Invasiveness:	B
Source of Animals:	(i) Client owned/donated, (ii) SPCA Selangor, (iii) Paws Animal Welfare Society (PAWS), (iv) Pusat Perlindungan Kucing Putrajaya (PPKP), (v) Pusat Kurungan Haiwan, Jabatan Kesihatan DBKL, (vi) Furry Friends Farms.
Number of Animals Approved:	50 cats
Housing:	Not Applicable
Duration:	20 July 2018 – 20 July 2019

Ethical approval is required in the case of amendments to the approved animal utilisation protocol (AUP). Please apply using Form 105. Kindly submit a final/annual report (Form 106) upon study completion, or before expiry of approval.

PROF. DR. MOHD HAIR BEJO
Chairman
Institutional Animal Care and Use Committee
Universiti Putra Malaysia

Pejabat Timbalan Naib Canselor (Penyelidikan dan Inovasi), Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
Pejabat Timbalan Naib Canselor (P&I) 603-8947 1002, Pejabat Pentadbiran TNCPI 603-8947 1608, Pejabat Pengarah, Pusat Pengurusan Penyelidikan (RMC) 603-8947 1601, Pejabat Pengarah, Putra Science Park (PSP) 603-8947 1291
http://www.tncpi.upm.edu.my

Appendix 2

Client Consent Form

FACULTY OF VETERINARY MEDICINE
UNIVERSITI PUTRA MALAYSIA (UPM)

Client Consent Form

Research Title: Urine collection from cats for the development of quantitative assay for feline morbillivirus

We are pleased to invite you and your companion animal to participate in a study to detect the presence of feline morbillivirus in cats. This research will increase our understanding of the presence of this newly-identified virus with the potential association with kidney disease. This research is closely supervised by our veterinarian, Dr Farina Mustaffa Kamal from UPM.

In order to conduct this study, we will only require a small amount of urine from your cat. All samples will be collected by an experienced registered veterinarian. Every measure will be taken to ensure that the collection of urine will be carried out in a stress-free environment and cause no distress to your pet(s).

Your participation is voluntary and you may withdraw anytime however, we greatly appreciate you and your pet(s) full participation in this study. Rest assured that any personal data shared with us will be held with utmost confidentiality. For further questions or concerns, you may contact for IACUC at 03-89471244 or our principal investigator at 03-86093466.

Thank you for your participation and making a difference to Malaysian cats and owners!

I hereby give my consent for urine collection from my cats by veterinarians and assistants.

Owner's name: …… Pet's name: …….
Case number: ……..

Signature: …… Date: …….

If you would like to be informed with the results of the screening, kindly provide us with the following details:

Email:…… Phone no.:……

BIODATA OF STUDENT

Siti Tasnim binti Makhtar was born on 28th August 1993 at Kota Bharu, Kelantan. She received her primary school education at Sekolah Rendah Islam (SRI) Al-Hikmah. Then she went to Maahad Muhammadi Perempuan, Kota Bharu for her secondary education and transferred to Kolej Islam Sultan Alam Shah, Klang after completing her Penilaian Menengah Rendah (PMR) examination. After completing her Sijil Pelajaran Malaysia (SPM) in 2010, she continued her study in Foundation in Science at Universiti Teknologi MARA (UiTM) Puncak Alam. Upon completion of her foundation's study, she enrolled for Bachelor in Science (Biology) Honours in 2012 at Universiti Teknologi MARA (UiTM) Shah Alam. In 2016, she pursued her master's degree study in Virology at Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM) under supervision of Dr Farina Mustaffa Kamal as her supervisor and being co-supervised by Professor Abdul Rahman Omar and Dr Tan Sheau Wei.

LIST OF PUBLICATIONS

Makhtar, S. T., Tan, S. W., Nasruddin, N. A., Abdul Aziz, N. A., Omar, A. B. and Mustaffa-Kamal, F. (2021). Development of Taqman-based real-time RT-PCR assay based on *N* gene for the quantitative detection of feline morbillivirus. *BMC Veterinary Research*, 17(1): 128

Siti Tasnim, M., Tan, S. W., Omar, A. R., Mustaffa-Kamal, F. (2021). Sequencing analysis of partial *N* gene of feline morbillivirus from Malaysia. *Journal of Tropical Agricultural Science*. (submitted)

Siti Tasnim, M., Mohd Isa, N. H., Omar, A.R., Selvarajah, G. T., Khor, K. H., Tan, S. Mustaffa-Kamal, F. (2019). Molecular detection and development of Taqman-based real-time RT-PCR (qRT-PCR) assay for the quantitative detection of feline morbillivirus from Malaysian domestic cats. In Proceedings of *MSAVA National Scientific Conference 2019, 22nd–23rd June 2019* at Connexion@Nexus, Kuala Lumpur, Malaysia.

Sifa, A. H., Yasmin, A. R., Noraniza, M. A., Mohammed, H. O., Omar, A. R., Arshad, S. S., Jalila, A., Ain-Najwan, M. Y., Ayuni, W. N. & **Siti Tasnim, M.** (2018). Serological and molecular detection of West Nile virus in equines in Malaysia. In *13th Proceedings of the Seminar on Veterinary Sciences, 20th – 22nd February 2018* at Faculty of Veterinary Medicine, Universiti Putra Malaysia, Selangor, Malaysia

Siti Tasnim, M., Muhammad Azlil, A., Abdul Rahman, O., Tan, S. W. and Farina, M. K. (2017). Characterisation of feline morbillivirus from Malaysian isolates. In Proceedings of *29th Veterinary Association Malaysia (VAM) Congress 2017, 6th–8th October 2017* at Holiday Inn Kuala Lumpur Glennmarie, Selangor, Malaysia.

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NAME OF STUDENT: SITI TASNIM BINTI MAKHTAR

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