Developing a detection method for rabies virus neutralizing antibodies using pseudotype technique

Miss Nichapat Rojjananavin

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Veterinary Pathobiology
Department of Veterinary Pathology
FACULTY OF VETERINARY SCIENCE
Chulalongkorn University
Academic Year 2021

การพัฒนาวิธีการตรวจหานิวทรัลไลซิ่งแอนติบอดีต่อไวรัสโรคพิษสุนัขบ้าโดยเทคนิคซูโดไทป์

น.ส.ณิชาภัทร โรจนนาวิน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาพยาธิชีววิทยาทางสัตวแพทย์ ภาควิชาพยาธิวิทยา
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2564

Thesis Title	Developing a detection method for rabies virus neutralizing antibodies using pseudotype technique
By	Miss Nichapat Rojjananavin
Field of Study	Veterinary Pathobiology
Thesis Advisor	Navapon Techakriengkrai, D.V.M., M.Sc., Ph.D.

Accepted by the FACULTY OF VETERINARY SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the FACULTY OF VETERINARY SCIENCE

(Professor SANIPA SURADHAT, D.V.M., Ph.D.)

THESIS COMMITTEE

..... Chairman

(Associate Professor THEERAYUTH KAEWAMATAWONG, D.V.M., Ph.D.)

..... Thesis Advisor

(Navapon Techakriengkrai, D.V.M., M.Sc., Ph.D.)

..... Examiner

(Associate Professor KANISAK ORAVEERAKUL, D.V.M., Ph.D.)

..... Examiner

(Associate Professor AUNYARATANA THONTIRAVONG, D.V.M., M.Sc., Ph.D.)

..... External Examiner

(Assistant Professor Suwarak Wannaratana, D.V.M., M.Sc., Ph.D.)

ณิชาภัทร โรจนนาวิน : การพัฒนาวิธีการตรวจหานิวทรัลไลซิ่งแอนติบอดีต่อไวรัสโรคพิษสุนัขบ้าโดยเทคนิคซูโดไทป์. (Developing a detection method for rabies virus neutralizing antibodies using pseudotype technique) อ.ที่ปรึกษาหลัก : ดร. นวพล เตชะเกรียงไกร

วิธีตรวจหาระดับของนิวทรัลไลซิ่งแอนติบอดีต่อไวรัสพิษสุนัขบ้า (rabies virus neutralizing antibody, RVNA) ที่ได้รับการยอมรับจากองค์การอนามัยโลกและองค์การสุขภาพสัตว์โลกในปัจจุบัน ได้แก่ การทดสอบเรพิดฟลูออเรสเซนท์โฟกัสอินฮิบิชั่น (rapid fluorescent focus Inhibition test; RFFIT) และการทดสอบฟลูออเรสเซนท์แอนติบอดีไวรัสนิวทรัลไลเซชั่น (fluorescent antibody virus neutralization test; FAVN) ซึ่งทั้งสองวิธีจำเป็นต้องใช้ไวรัสพิษสุนัขบ้าที่มีชีวิตและสามารถก่อโรคได้จึงอาจเป็นอันตรายต่อผู้ปฏิบัติงาน ทำให้วิธีดังกล่าวมีค่าใช้จ่ายสูงและใช้เวลานาน การศึกษานี้จึงทำการพัฒนาซูโดไทป์ของไวรัสพิษสุนัขบ้าขึ้นมาเพื่อใช้แทนไวรัสที่มีชีวิตและสามารถอ่านผลได้ภายใน 48 ชั่วโมง โดยพบว่าซูโดไทป์ที่สร้างจากระบบเลนติไวรัส (lentivirus) ให้ปริมาณไวรัสสูงกว่าระบบวีเอสวี (vesicular stomatitis virus, VSV) และพบว่าวิธีการตรวจหานิวทรัลไลซิ่งแอนติบอดีต่อไวรัสพิษสุนัขบ้าโดยเทคนิคซูโดไทป์ในตัวอย่างซีรั่มสุนัขทั้งหมด 50 ตัวอย่าง (n=50) มีความไว (sensitivity) และความจำเพาะ (specificity) ที่ร้อยละ 92 และร้อยละ 100 ตามลำดับ โดยซูโดไทป์ของไวรัสพิษสุนัขบ้าที่สร้างขึ้นนั้นไม่ทำปฏิกิริยาข้ามกับโมโนโคลนอลแอนติบอดีที่จำเพาะต่อไวรัสไข้หัดสุนัข และแสดงความสามารถในการทวนซ้ำ (repeatability) ที่สูงด้วยค่าสัมประสิทธิ์ของความแปรปรวน (coefficient of variation) เพียง 1.33 นอกจากนี้ เมื่อเปรียบเทียบกับวิธีมาตรฐาน FAVN ยังพบว่าระดับ RVNA ที่อ่านได้จากทั้งสองวิธีสอดคล้องกันอย่างมาก ด้วยค่าสัมประสิทธิ์ Pearson r เท่ากับ 0.9491 (p value < 0.0001) จึงกล่าวได้ว่าการใช้เทคนิคซูโดไทป์นอกจากเพิ่มความปลอดภัยต่อผู้ปฏิบัติงานและใช้เวลาในการตรวจที่สั้นลงแล้ว ยังเป็นวิธีการตรวจที่สอดคล้องกับวิธีมาตรฐาน FAVN วิธีการตรวจหานิวทรัลไลซิ่งแอนติบอดีต่อไวรัสพิษสุนัขบ้าโดยเทคนิคซูโดไทป์ที่พัฒนาขึ้นนี้นับเป็นทางเลือกหนึ่งที่มีศักยภาพสำหรับการตรวจสถานะทางภูมิคุ้มกันในสุนัขในอนาคต

สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2564 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

6175304031 : MAJOR VETERINARY PATHOBIOLOGY

KEYWORD: fluorescent antibody virus neutralization test, rabies virus, rabies virus neutralizing antibody, rabies virus pseudotype, serum neutralization test

Nichapat Rojjananavin : Developing a detection method for rabies virus neutralizing antibodies using pseudotype technique. Advisor: Navapon Techakriengkrai, D.V.M., M.Sc., Ph.D.

Rabies is a neuro-fatal disease, causing by rabies virus (RABV) infection. Two serological tests, recommended by the World Health Organization and the World Organization for Animal Health, namely the rapid fluorescent focus inhibition test (RFFIT) and the fluorescent antibody virus neutralization test (FAVN) are considered gold standard. However, as both the RFFIT and FAVN require the use of live viruses, they raise biosafety concerns. Moreover, the immunostaining step in both methods is costly and time-consuming. In this study, RABV-pseudotype was developed and used in a RVNA detection method. The RABV-pseudotype based on lentivirus gave higher titer than the vesicular stomatitis virus (VSV). Fifty dog serum samples were tested for RVNA titer and compared with FAVN to validate the new pseudotypre-based method. The diagnostic sensitivity and specificity of this method was 92% and 100%, respectively. The analytical specificity of the test was confirmed by lacking of cross-neutralization with an anti-CDV monoclonal antibody. The test repeatability was demonstrated by the coefficient of variation of 1.33 among 4 different timepoints. The RVNA titer measured by both methods was in a strong positive correlation (Pearson $r = 0.9491$, $p < 0.0001$). In conclusion, the RABV pseudotype-based assay developed in this study offers a safer and faster means for assessing the immune status of the dog population.

Field of Study: Veterinary Pathobiology Student's Signature .....

Academic Year: 2021 Advisor's Signature .....

ACKNOWLEDGEMENTS

First, I would like to express my deepest gratitude to my advisor Dr. Navapon Techakriengkrai for providing me the knowledge and suggestions to complete this study. Also, I would like to thank my committees, Assoc. Prof. Theerayuth Kaewamatawong, Assoc. Prof. Kanisak Oraveerakul, Assoc. Prof. Aunyaratana Thontiravong, and Asst. Prof. Suwarak Wannaratana for their advice. I also thank members of the Virology Unit and Microbiology Department, Faculty of Veterinary Science for their help. I also thank the National Institute of Animal Health (NIAH), Department of Livestock Development, Ministry of Agriculture and Cooperative, for kindly providing the samples used in this study. Finally, I would like to thank Chulalongkorn University graduate scholarship to commemorate the 72nd anniversary of his Majesty King Bhumibol Aduladej and CU Graduate School Thesis Grant

Nichapat Rojjananavin

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

Introduction

Rabies virus (RABV), which belongs to the genus *Lyssavirus* within the family *Rhabdoviridae* (Johnson et al., 2010) is a causative agent of rabies, a neuro-fatal disease in almost all mammals. The major reservoir for RABV is dog, as a result, almost all human cases are reportedly from dog bites. RABV in dog's saliva enter the wound, replicate and migrate centripetally to the central nervous system (CNS). Once RABV reach CNS, infected hosts show the clinical sign including abnormal behavior, hypersensitivity to light and sound, hypersalivation, dysphagia, and hydrophobia (Fooks et al., 2017). To date, the most effective prevention recommended by the world health organization (WHO) is pre-exposure prophylaxis (PrEP) with RABV vaccination and post-exposure prophylaxis (PEP) with rabies immunoglobulins (RIG) (World Health, 2018). Nevertheless, rabies fatalities remain relatively high, with an approximation of 59,000 deaths annually, mostly in Africa and Asia (Fooks et al., 2017). In Thailand, Rabies is still one of the major public health concerns, with a large number of cases reported in the central part of Thailand, especially in Bangkok (Kasempimolporn et al., 2007). Bangkok has a large stray dog population and lack of appropriate management (Kasempimolporn et al., 2011). To successfully control rabies transmission, at least 70% of the dog population should be vaccinated (WHO, 2005). In addition to vaccine coverage, another important attribute for rabies control is the induction of neutralizing antibody (RVNA) in which the titer of at least 0.5 IU/ml is required for a complete protection. Even though an annual rabies vaccination program is practiced in Thailand, the efficacy of the program is rarely assessed partly due to the limited number of testing facility and high cost.

The trimeric envelope glycoprotein (G protein) of rabies virus is responsible for virus entry into host cell and, therefore a main target for rabies virus neutralizing antibody (RVNA) (Johnson et al., 2010). Currently, there are two gold-standard methods for RVNA quantification respectively recommended by WHO (World Health

Organization) and OIE (World Organization for Animal Health) namely, a rapid fluorescent focus Inhibition test (RFFIT) (Smith et al., 1973) and a fluorescent antibody virus neutralization test (FAVN) (Cliquet et al., 1998). Both the RFFIT and FAVN base on the principle of serum neutralization test (SN test), which quantify the RVNA titer against RABV CVS-11 strain (rabies challenge virus standard strain, CVS-11). Serum is first serially-diluted and incubated with RABV for one hour before transferred into an 8-well chamber slide pre-seeded with BHK-21 cells for RFFIT or a 96-well plate for FAVN. Afterward, infected cells are detected using a fluorescein isothiocyanate (FIT-C) conjugated anti-RABV nucleoprotein antibodies under a fluorescent microscope and converted to RVNA titer (Burgado et al., 2018). However, as both the RFFIT and FAVN require the use of live viruses, they raise biosafety and biosecurity concerns (OIE, 2018a). In addition, the immunostaining step in both tests is costly and time-consuming. On the contrary, an antigen-binding assay or an enzyme-linked immunosorbent assay (ELISA) is safer and more robust (Wasniewski and Cliquet, 2012). However, this assay might not reflect the true level of protection (De Benedictis et al., 2012).

To address the aforementioned issues with RFFIT and FAVN, the objective of this study is to develop a new RABV serum neutralizing test using a pseudotype technique. Pseudotype is a recombinant viral particle that carries envelope proteins of one virus on the core of another. As some essential genes of the viral core are removed from its genome, pseudotype can only undergoes a single round of replication and considered safe (Li et al., 2018). In this study, an envelope glycoprotein gene of the RABV CVS-11 strain was used to produce the RABV-pseudotype based on two viral backbones, namely vesicular stomatitis virus (VSV) and lentivirus (HIV). In addition, as these pseudotypes encode green fluorescent protein (GFP) gene in their genome, the immunofluorescent staining step is no longer required. The RABV-pseudotype based assay offers several advantages over RFFIT and FAVN, including increased biosafety, faster turnaround time and more economic.

Hypothesis

1. RABV pseudotype can be developed and used for the detection of rabies virus neutralizing antibodies.

2. The rabies virus neutralizing antibody (RVNA) titers quantified by this new serum neutralization test correlates with titer from the fluorescent antibody virus neutralization (FAVN) assay.

Expected benefits

A pseudotype based SN-test for rabies virus neutralizing antibody quantification

Conceptual framework

Developing a detection method for rabies virus neutralizing antibodies using pseudotype technique

1. RABV-pseudotype production

1.1 vesicular stomatitis virus system

1.1.1 Producer cells and plasmid concentration optimization

1.1.2 MOI, time, and target cells optimization

1.2 Lentivirus system

1.2.1 Production of RABV-lentiviral pseudotype

1.2.2 Target cells selection

1.3 RABV-pseudodotype titer comparison

Comparison of viral titer between the VSV system and the lentivirus system

2. serum neutralization (SN) test optimization

2.1 Infection method and target cells selection

2.2.1 Adsorption

2.2.2 Co-culture

2.2 Cut off determination

IC100 and IC90 cut off selection

3. Quantification of RVNA titer by SN-test

Comparison of RVNA titer between SN-test and FAVN test (n=50)

4. SN test validation and statistical analysis

4.1 Analytical specificity

4.2 Diagnostic sensitivity and specificity

4.3 Repeatability

4.4 Correlation of RVNA titer determined by SN-test and FAVN

Outcome

A serum neutralization test for RVNA quantification that correlates with titer from a fluorescent antibody virus neutralization (FAVN) assay

CHAPTER 2

Objective

To develop a rabies virus serum neutralization test using pseudotype technique

CHAPTER 3

Literature review

Rabies epidemiology in Thailand

Rabies is an ancient neuro-fatal disease caused by rabies virus (RABV) infection. In Thailand, rabies is endemic and remains a public health concern, with dogs as a major reservoir (Komol et al., 2020). Molecular genetic analysis of RABV from animal and human samples found that RABV in Thailand belongs to the same lineage of South-east Asia (SEA) and China (Benjathummarak et al., 2016). Moreover, both RABV G genes and N gene analyses showed that all Thai RABV isolates belong to lyssavirus genotype I, and further separate into 2 clades, namely THA-1 and THA-2 (Denduangboripant et al., 2005; Benjathummarak et al., 2016). THA-1 was found in the central part of Thailand whereas THA-2 was distributed in almost every provinces in the northeastern region (Denduangboripant et al., 2005). Clade THA-1 is further divided into 2 subclades, namely THA-1A and THA-1B, respectively (Benjathummarak et al., 2016).

Rabies virus (RABV)

1. Lyssavirus genus classification

Rabies virus belongs to genus *Lyssavirus*, family *Rhabdoviridae* of the order Mononegavirales (Fooks et al., 2017). The genus *Lyssavirus* are classified by the international committee on taxonomy of viruses (ICTV), based on the genetic distance into 12 species. These lyssaviruses have been divided into three phylogroups with distinct pathogenicity and immunogenicity (Weir et al., 2014) (Table 1). The phylogroup I includes Rabies virus (RABV), Duvenhage virus (DUVV), European bat lyssavirus 1 (EBLV1), European bat lyssavirus 2 (EBLV2), Australian bat lyssavirus (ABLV), Khujand virus (KHUV), and Irkut virus (IRKV). RABV vaccine confers cross-neutralization and protection against all members of the phylogroup I but only little or no cross-protection against the members of phylogroup II (Mokola virus (MOKV),

Lagos bat lyssavirus (LBV), and Shimoni bat virus (SHIBV)) (OIE, 2018b). However, no cross-reactivity between the phylogroup III (West Caucasian bat virus (WCBV), Ikoma lyssavirus (IKOV) and Lleida bat lyssavirus (LLEBV)) and the phylogroup I and II (Fooks et al., 2014). Unclassified species includes Bokeloh bat lyssavirus (BBLV), Gannorura bat lyssavirus (GBLV), Taiwan bat lyssavirus (TBLV), Ikoma lyssavirus (IKOV), Lleida bat lyssavirus (LLEBV) and Kotalahti bat lyssavirus (KBLV).

Table 1 Lyssaviruses classification

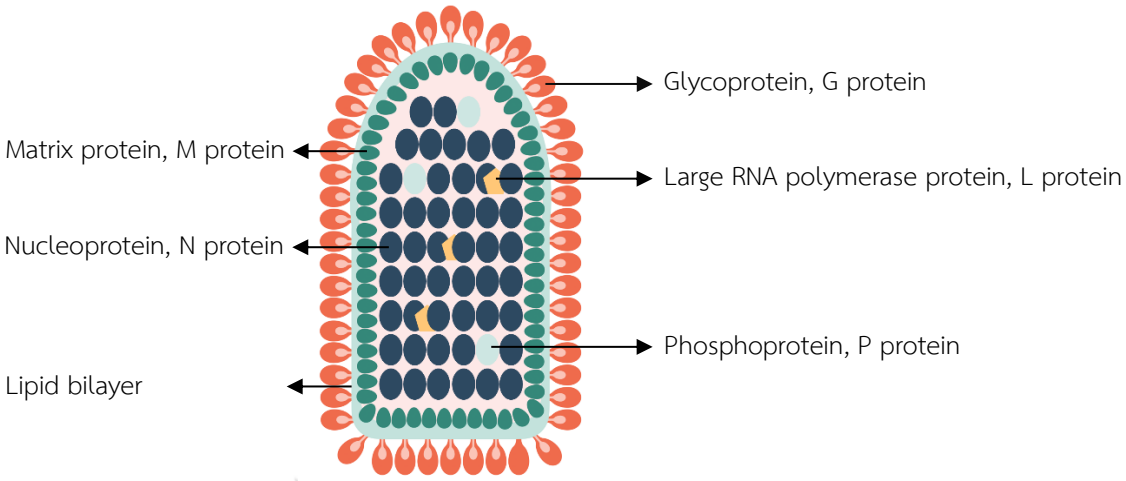
Phylogroup	Genotype	Virus	
I	1	Rabies virus	RABV
	4	Duvenhage virus	DUVV
	5	European bat lyssavirus type 1	EBLV1
	6	European bat lyssavirus type 2	EBLV2
	7	Australian bat lyssavirus	ABLV
	8	Aravan virus	ARAV
	9	Khujand virus	KHUV
	10	Irkut virus	IRKV
	NC*	Bokeloh bat lyssavirus	BBLV
	NC	Gannorura bat lyssavirus	GBLV
	NC	Taiwan bat lyssavirus	TBLV
II	2	Lagos bat lyssavirus	LBV
	3	Mokola virus	MOKV
	12	Shimoni bat virus	SHIBV
III	11	West Caucasian bat virus	WCBV
	NC	Ikoma lyssavirus	IKOV
	NC	Lleida bat lyssavirus	LLEBV
NC	NC	Kotalahti bat lyssavirus	KBLV

*NC = not yet classified by the International Committee for the Taxonomy of Viruses)

2. Viral structure and genome organization

Rabies virus (RABV) is an enveloped virus with a bullet-shape morphology. RABV genome is non-segmented, negative-sense, single strand RNA. RABV genome is approximately 12 kilobase in length and encodes five viral proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA dependent RNA polymerase (L). Nucleoproteins encapsidate new genomic RNA into ribonucleoprotein (RNP) complex, which protect genomic RNA from degradation by cellular RNases (Fisher et al., 2018). The phosphoprotein is the non-catalytic subunit of the polymerase complex, that provide the connection between the RNP and polymerase protein. Moreover, the phosphoprotein antagonizes innate immunity in the infected host. The matrix proteins surround the RNP and located next to the viral envelope, connecting with the carboxy-terminal region of the glycoprotein (Realegeno et al., 2018). The glycoprotein forms trimers that responsible for binding with the host cell receptor and is the major surface antigen target of rabies neutralizing antibodies (Fooks et al., 2014).

a)



b)

Figure 1 The structure of rabies virus (RABV) and genome organization. a) the structure of rabies virus and viral protein component including Nucleoprotein (N), Phosphoprotein (P), Matrix protein (M), Glycoprotein (G), Large RNA polymerase protein (L). b) RABV genome organization.

3. viral replication

After host cell attachment via glycoprotein (G), RABV enters the cell by receptor-mediated endocytosis (Weir et al., 2014). Subsequently, an acidic environment of the endosome induces conformational change of the glycoprotein that mediates fusion of the viral envelope with the cellular membrane (Albertini et al., 2011). Then, viral genome is released into the cytoplasm (Rampersad and Tennant, 2018). After released, the negative-stranded genomic RNA is converted by viral RNA dependent RNA polymerase (RdRp) complex into a positive stand for genomic RNA replication and viral protein production (Fodor, 2020). For replication, the full-length, positive sense anti-genomic strand serves as template for the synthesize of the nascent full length, negative-sense RNA strands (Fooks et al., 2017). For transcription, 5 mRNAs with leader sequence, 5' cap and 3' poly(A) tail are synthesized by a gradient-based stop-start mechanism in the following order: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and polymerase (L) (Albertini et al., 2011).(Fisher et al., 2018). Viral proteins are translated on free ribosomes in the cytoplasm except for glycoprotein, which occur in endoplasmic reticulum (ER) and Golgi apparatus and then transported to plasma membrane (OIE, 2018a). Finally, viral components are assembled and bud out of the cell as a new virion.

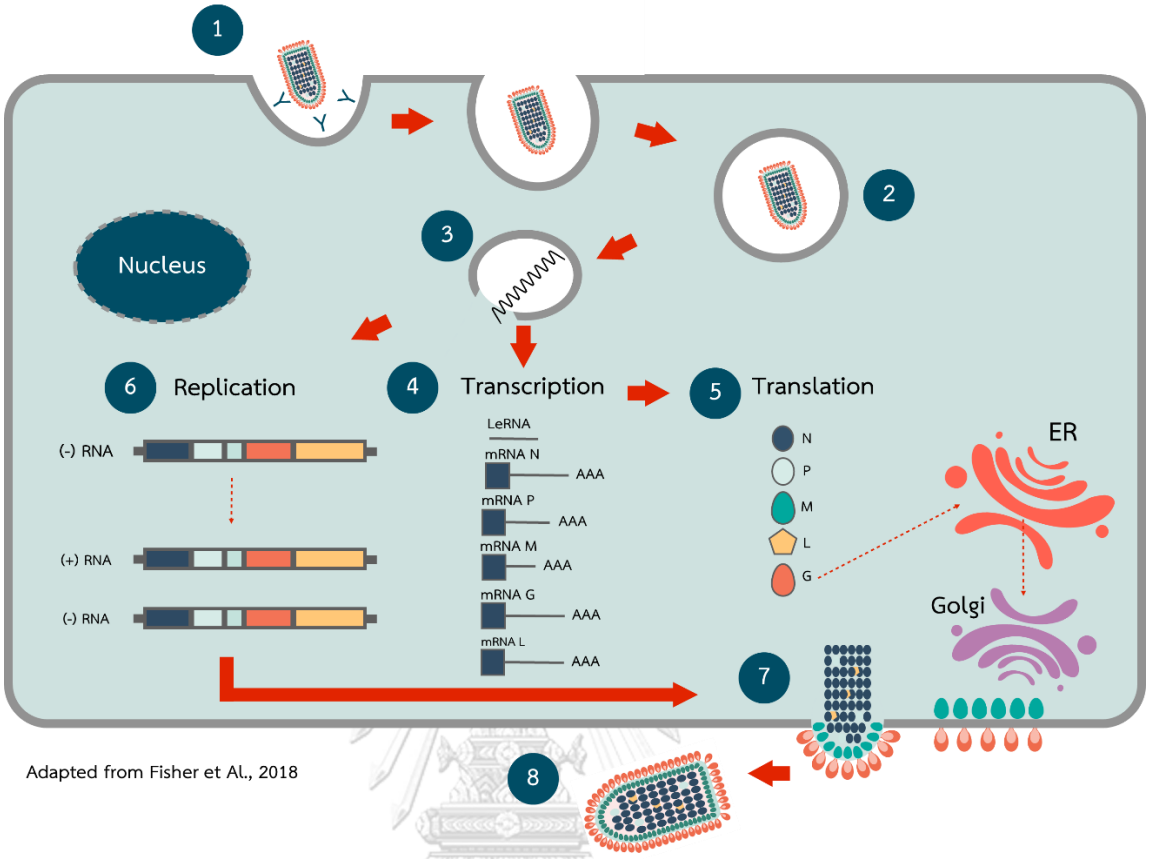


Figure 2 Rabies virus replication cycle 1) attachment, 2) endocytosis, 3) uncoating, 4) transcription, 5) translation, 6) genome replication, 7) assembly, 8) budding.

Serological method for RABV specific antibody detection

The serological test for RABV specific antibody plays a crucial role for the assessment of vaccine-induced immune status, especially in pets for international travel, and oral vaccine efficacy in wildlife (OIE, 2018a). Two gold standard methods recommended by WHO (World health organization) and OIE (World organization for animal health) including FAVN (fluorescent antibody virus neutralization test) (Cliquet et al., 1998) and RFFIT (rapid fluorescent focus inhibition test) (Smith et al., 1973) are developed to replace the MNT (mouses neutralization test) method. Both the FAVN and RFFIT, are *in vitro* quantitative method detecting the RABV neutralizing antibody (RVNA) level in serum sample. RFFIT is a semi-quantitative method that is performed in an 8-well chamber slide. The fluorescent foci or RABV-infected cell are then observed in 20 microscopic fields in each well (Burgado et al., 2018). On the contrary, FAVN is performed in a 96-well plate similar to other conventional serum-neutralization tests. However, both assays require immunofluorescent staining of RABV-N protein in the infected cell to determine virus infectivity, making them costly and labor intensive. In addition, the use of live virus also creates biosafety and biosecurity concerns (OIE, 2018b). Antigen-binding assay such as, ELISA (enzyme-link immunosorbent assay) is more rapid and does not require using of live-virus and cell culture equipment (Servat et al., 2007). Usually, ELISA detects RABV-specific antibodies in serum by using the RABV glycoprotein or nucleoprotein coated plate. Moreover, ELISA can distinguish different immunoglobulin subtypes (IgG and IgM) and commercially available as both the indirect ELISA and competitive ELISA format. Nevertheless, the RABV-specific antibodies detected by ELISA do not represent the level of protection (De Benedictis et al., 2012) Therefore, neutralization assays remains an important means for the detection of RVNA level (OIE, 2018a).

Table 2 Comparison of FAVN and RFFIT tests

Test	Perform	Reading result	Turnaround time	Advantage	Disadvantage
FAVN[a]	96 well plate	Total surface of each well observed	4-5 Days	High sensitivity and specificity for RVNA quantification	Time-consuming, require live virus, labor-intensive
RFFIT[b]	8 well chamber slide	Random 20 fields observed	3-4 Days		

[a]) FAVN; the fluorescent antibody virus neutralization test

[b]) RFFIT; the rapid fluorescent focus inhibition test

RABV-pseudotype

Pseudotype is a recombinant virus composing of a core of one virus and envelope glycoproteins of another. Pseudotype virus is widely used for the serological study of highly pathogenic viruses and zoonotic diseases as it can undergoes only a single round of replication cycle and can be handled in the biosafety level 2 laboratories (Li et al., 2018). Several viral cores have been developed and used for pseudotype production including vesicular stomatitis virus (VSV), a Rhabdovirus, and lentivirus. In the VSV system, the glycoprotein gene of the VSV is deleted and replaced with reporter gene such as green fluorescent protein (GFP) or luciferase (Whitt, 2010). In the lentivirus system, essential genes are separated into 3 plasmids, namely packaging plasmid, envelope plasmid and transgene plasmid, which encode lentiviral structural proteins, envelope glycoproteins and reporter gene, respectively (Toon et al., 2021). To produce the RABV-pseudotype, the envelope glycoprotein (G) of RABV is provided *in trans* by transfection to the VSV core or lentiviral core. Previous study utilizing lentiviral core reported that the RABV-pseudotype provided reliable outcomes for serological study (Wright et al., 2008). However, critical points for pseudotype production including using of different viral core, producer cell type, and expression plasmids, must be optimized to achieve maximal viral titer and a pseudotype particle that resemble functional glycoprotein of the live virus (Li et al., 2018).

CHAPTER 4

Materials and Methods

Cell lines

1. BHK-21 C13 cell
2. HEK 293T cell
3. HEK 293FT cell
4. Vero E6 cell

Baby hamster kidney (BHK-21) clone 13 cell, a standard target cell for rabies virus infection was maintained in Opti-MEM™ Reduced Serum Medium (Gibco®, Life Technologies, Carlsbad, CA) supplemented with 2% heat-activated fetal bovine serum (FBS), 2mM L-glutamine and gentamicin (40 mg/ml). Human embryonic kidney (HEK) 293T cell and Human embryonic kidney 293FT cell, conventional cell lines for viral propagation, were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% heat-activated fetal bovine serum (FBS), 2mM L-glutamine, 10,000 units/ml of penicillin, 10,000 µg/ml of streptomycin, and 25 µg/ml of Gibco Amphotericin B. (Antibiotic-Antimycotic (100X), Gibco®). Vero E6 (Vero 76, clone E6 (European Culture of Authenticated Cell cultures (ECACC), Salisbury, UK, 85020206), another commonly used target cell for rabies virus, was maintained in Modified Eagle's Medium (MEM) with 10% heat-activated fetal bovine serum (FBS), 2mM L-glutamine, 10,000 units/ml of penicillin, 10,000 µg/ml of streptomycin, and 25 µg/ml of Gibco Amphotericin B. (Antibiotic-Antimycotic (100X), Gibco®).

Viruses and plasmids

1. Recombinant virus G*rVSVΔG-GFP
2. Plasmid pCAG RABV-G
3. Plasmid pCCGW
4. Plasmid pSPAX2

The recombinant virus G*rVSVΔG-GFP, was used for the VSV-pseudotype production. The recombinant virus carries a full-length VSV genome in which the glycoprotein (G) gene was replaced with the reporter gene, green fluorescent protein (GFP). The plasmid pCAG RABV-G encoded the RABV-CVS11 glycoprotein gene. The plasmids pCCGW and pSPAX2 are second generation lentiviral vectors, encoded the green fluorescent protein (GFP) gene and structural proteins of human immunodeficiency virus (HIV), respectively under CMV promoter. Together with the plasmid pCAG RABV-G, recombinant virus G*rVSVΔG-GFP was used for the VSV-based pseudotype production, whereas the plasmids pCCGW and pSPAX2 were used for the lentiviral-pseudotype production.

Table 3 Detail of the recombinant agents used in the study

Recombinant agents	Function	Source
Plasmid pCAG RABV-G	expressing envelope glycoprotein of RABV strain CVS-11	a gift from Connie Cepko (Addgene plasmid #36398)
Plasmid pCCGW	encoding the green fluorescent protein gene (GFP)	Navapon Techakriengkrai (University of Glasgow, 2016)
Plasmid pSPAX2	expressing lentiviral structural proteins and essential enzyme	a gift from Didier Trono (Addgene plasmid #12260)

Reference serums

The OIE Standard serum of dog origin, batch number 2014-1 (OIE Reference Laboratory for Rabies, Nancy, France) was resuspended according to the manufacturer with phosphate buffered saline (PBS) to 0.5 IU/ml. A dog serum naïve for rabies virus, batch number SR-15 (OIE Reference Laboratory for Rabies, Nancy, France) was used as a negative reference control.

Table 4 Detail of the reference serum used in the study

Reference serum	Function	Source
The OIE standard serum of dog origin (batch number 2014-1)	Positive control	OIE Reference Laboratory for Rabies, Nancy, France
A dog serum naïve for rabies virus (batch number SR-15)	Negative control	OIE Reference Laboratory for Rabies, Nancy, France

Monoclonal antibodies

The anti-RABV monoclonal antibody, clone E559 (a gift from Dr. Waranyoo Phoolcharoen) (1.35 mg/µl) was used for an internal control and for repeatability testing. The anti-CDV monoclonal antibody, clone 95J4 (Creative Diagnostic®, New York, USA) was used for analytical specificity testing.

Table 5 Detail of the monoclonal antibodies used in the study

Monoclonal antibodies	Function	Source
anti-RABV monoclonal antibody, clone E559 (1.35 mg/µl)	internal control and repeatability testing	a gift from Dr. Waranyoo Phoolcharoen
anti-CDV monoclonal antibody, clone 95J4	analytical specificity testing	Creative Diagnostic®, New York, USA

Serum samples

Fifty dog serum samples (n=50), previously tested for anti-RABV titer by FAVN, were kindly provided from the National Institute of Animal Health, Department of Livestock Development, Ministry of Agriculture and Cooperative. All of serum samples were heat inactivated at 56°C for 30 minutes and stored frozen at -20°C until testing.

Table 6 Detail of serum samples used in the study

Serum samples	Source
50 dog serum samples (n=50), previously tested for anti-RABV titer by FAVN	kindly provided by the National Institute of Animal Health

Part 1: RABV-pseudotype production

The productions of RABV-pseudotype based on both the vesicular stomatitis virus (VSV) and the lentivirus system were explored in this study.

1.1 vesicular stomatitis virus system

To produce the VSV(RABV) pseudotype, the various critical step that affects the viral titer such as the producer cell and DNA plasmid concentration for transfection step, the amount of recombinant virus, and incubation time for superinfection step and target cell susceptible. In this study, the producer cell and plasmid concentration were optimized followed by MOI, time, and target cells optimization.

1.1.1 Producer cells and plasmid concentration optimization

To optimize the VSV(RABV) pseudotype production, the HEK293T and HEK293FT cells were used as producer cells. Five hundred thousand cells/ml of the producer cells were plated into a 10 cm tissue-culture dish and incubated overnight at 37°C, 5% CO_2. After incubation, the cells were transfected with pCAG RABV-G at different concentration (12 or 16 µg/dish) by using polyethyleneimine (PEI) at 1:3 (DNA:PEI) ratio and incubated for 4 hours. Then, the medium was removed and replaced with 10 ml of fresh DMEM cell culture medium. Next day, the cells were superinfected with the recombinant virus (G*rVSVΔGFP) at Multiplicity of infection (MOI) of 1 or 3. Following 1-hour incubation cells were washed three times with

phosphate buffer saline (PBS) and replenished with fresh 10 ml of DMEM cell culture medium. After a period of 24 or 48 hours incubation at 37℃ with 5% CO_2, the cell culture supernatant that contains the RABV-pseudotype virus were harvested and filtered through 0.45 µM syringe filter, aliquoted, and stored at -80℃. The viral titer was determined by Spearman and Karber method (Spearman, 1908; Kärber, 1931) in Vero E6 cell lines. The producer cell and plasmid concentration that provided the highest titer was chosen for this study.

Table 7 Producer cell and plasmid concentration for the VSV(RABV)-pseudotype production

Producer cell	Plasmid and transfecting agent	Plasmid concentration (µg)
HEK293T / HEK293FT	Plasmid pCAG RABV-G	12
	Polyethyleneimine (PEI)	36
	Plasmid pCAG RABV-G	16
	Polyethyleneimine (PEI)	48

1.1.2 MOI, time, and target cells optimization

After the producer cell and plasmid concentration optimization step, HEK293FT was transfected with pCAG RABV-G at 16 µg before superinfected with a recombinant VSVΔG-GFP virus at MOI of 1 and 3. After 24 and 48 hours of incubation the supernatant was collected and determined the titer in BHK-21 and Vero E6 cell lines. Then, calculated as $TCID_{50}$ using Spearman and Karber method (Spearman, 1908; Kärber, 1931).

Table 8 MOI, time, and target cells optimization for VSV(RABV)-pseudotype production

Producer cell	MOI	Incubation time (hours)	Target cell
HEK293FT	MOI = 1	24	BHK-21 / VERO E6
		48	
	MOI = 3	24	
		48	

1.2 Lentivirus system

1.2.1 Production of RABV-lentiviral pseudotype

One day before transfection, 5 × 10^5 HEK293T cells were plated on the 10 cm tissue-culture dishes and incubated at 37°C, 5% CO_2. Transfection mixture composing of pCCGW, pSPAX2 and pCAG RABV-G at 8:4:4 ratio, 2 ml of serum-free Opti-MEM and 48 ug of PEI were mixed and incubated for 20 minutes at room temperature before transfection. The plasmids concentration 1:3 (DNA:PEI) ratio was shown in table 1. After 4 hours post transfection, 8 ml of DMEM was added to the cell culture dish and incubated for 48 hours at 37°C with 5% CO_2. The supernatant was collected by using a 0.45 µM syringe filter and stored at -80°C.

Table 9 Plasmid concentration for RABV-lentiviral pseudotype production

Producer cell	Plasmid and component	Plasmid concentration (µg)
HEK293T	Plasmid pCAG RABV-G	4
	Plasmid pCCGW	8
	Plasmid pSPAX2	4
	Polyethyleneimine (PEI)	48

1.2.2 Target cells selection

The RABV-lentiviral pseudotype was titrated on BHK-21, HEK293T and Vero E6 cell lines and calculated as $TCID_{50}$ using Spearman and Karber method (Spearman, 1908; Kärber, 1931).

1.3 RABV-pseudodotype titer comparison

The highest viral titer of RABV pseudotype produced by the VSV system and the lentivirus system were compared to select the optimal viral system and target cell for this study.

Part 2: serum neutralization (SN) test optimization

The serum neutralization assay (SN) is the serological method for detect the reaction between neutralizing antibody and virus by using cell culture technique. Also, the infection method and target cell should be optimized. Moreover, the cut-off for determined the RVNA titer can affect the result interpret and should be select carefully.

2.1 Infection method and target cell selection

To optimize the serum neutralization (SN) test, the infection method was first compared between the adsorption and co-culture techniques using 4 serum samples with known RABV neutralizing antibody (RVNA) titer at very low, middle, and super high titer (No.4, 7, 13, and 15) in BHK-21 and HEK293T cells. All methods were performed and tested on the same time to avoid the variation of freeze-thaw sample, cell passage number, and virus stock.

2.1.1 Adsorption method

For the adsorption method, 4×10^5 cells/ml of BHK-21 and HEK293T cells were plated in 96-well cell culture plate 24 hours before infection. For the control plate (Figure 3A), 50 µl of OIE serum, negative serum, and E559 RABV monoclonal antibody (1:500) were serially diluted 3-fold with 100 µl of cell culture medium (Opti-MEM for BHK-21 cells and DMEM for HEK293T cells) in 96-well cell culture plate. For the test plate (Figure 3B), 50 ul of serum sample also was serially diluted 3-fold with 100 µl of cell culture medium. Then, 100 $TCID_{50}$/50 ul of RABV-pseudotype virus was

added into each well and incubated for 1 hour at 37°C, 5% CO_2. After incubation 50 µl of cell culture medium was added each well and transferred to the target cell plate. The control plate and the test plate were incubated for 48 hours at 37°C, 5% CO_2 and observed the green fluorescent signal under an inverted fluorescent microscope.

2.1.2 Co-culture method

For the co-culture method, all serum controls and serum samples were serially diluted 3-fold similar to the adsorption method. Then, 100 $TCID_{50}$/50µl of RABV-lentiviral pseudotype was added into each well and incubated for 1-hour at 37°C, 5% CO_2. After the incubation, 50 µl of 4×10^5 cells/ml BHK-21 and HEK293T cells suspension was added into each well. The green fluorescent signal was observed under an inverted fluorescent microscope at 48 hours post-infection.

2.2 Cut-off determination

Similar to the FAVN method, total surface area of each well was observed for the green fluorescent signal under an inverted fluorescent microscope. The number of wells with 90% reduction in green fluorescent signal (IC90, inhibitory concentration 90), comparing to negative serum control, or a complete absence of green fluorescent signal (IC100) was first counted and converted into RVNA titer in IU/ml unit

$$\text{Serum titer (IU/ml)} = \frac{[(10^{(\text{serum log D50 value})}) \times \text{theoretical titer of OIE serum 0.5 IU/ml}]}{(10^{(\text{log D50 of OIE serum 0.5 IU/ml})})}$$

Then, the RVNA titer measured by IC90 or IC100 was compared to FAVN titer to determine the cut-off for the SN-test.

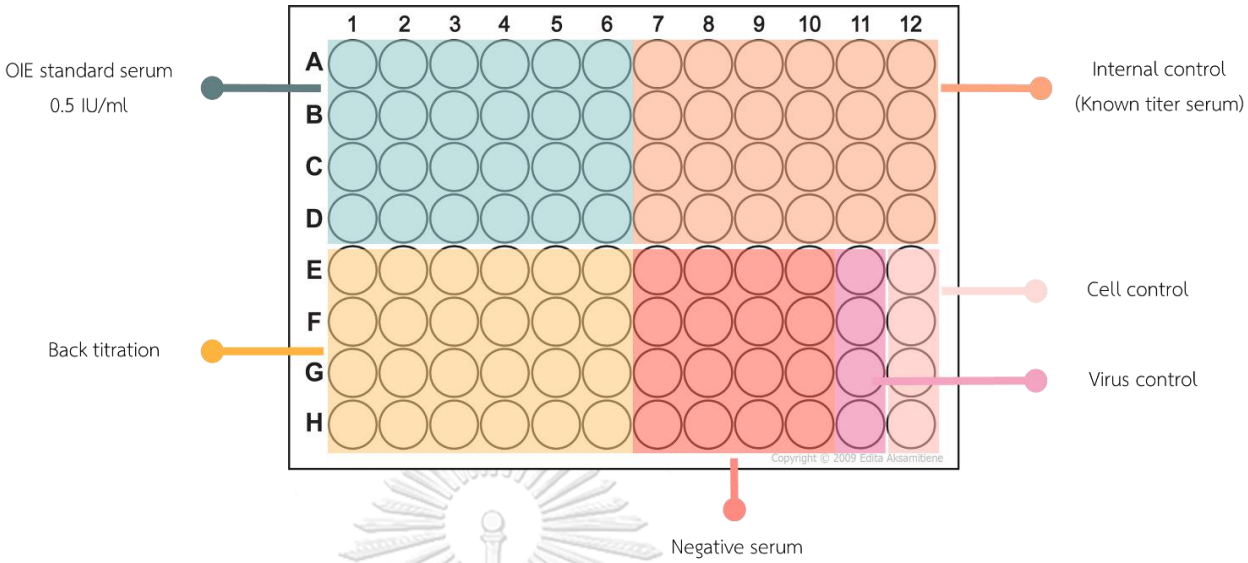
Part 3: Quantification of RVNA titer by SN-test

The control plate for the SN test was adapted from the FAVN method (OIE, 2018b) with a total number of 5 controls, including an OIE standard serum at concentration 0.5 IU/ml., the anti-RABV monoclonal antibody, clone E559 (1:500) as an internal positive control, the naïve dog serum control, the virus control, and the cell control. In addition, a back titration of the pseudotype was performed to confirm the titer of 100 $TCID_{50}$/50 ul. Fifty microliter of OIE standard serum, E559 and naïve dog serum was serially diluted 3-fold in 100 µl of DMEM. Fifty microliter of RABV pseudotype and DMEM was added into each well of the virus control and cell control, respectively. Fifty microliter of RABV pseudotype was serially diluted 3-fold in 100 µl of DMEM for back titration. Each test plate was used for 4 samples as shown in figure 3B. The serum samples were prepared by performing 3-fold serial dilution with 100 ul of DMEM and incubated with 100 $TCID_{50}$/50µl of the RABV pseudotype for 1 hour at 37°C, 5% CO_2. After incubation, 4×10^5 cells/ml of HEK293T cells suspension was added into each well and incubated for 48 hours at 37°C, 5% CO_2. The total surface of each well was observed under an inverted fluorescent microscope. The neutralizing antibody titer was determined by counting the number of well with complete absence of green fluorescence signal and converted into IU/ml using the following formula I:

$$\text{Serum titer (IU/ml)} = \frac{[(10^{(\text{serum log D50 value})}) \times \text{theoretical titer of OIE serum 0.5 IU/ml}]}{(10^{(\text{log D50 of OIE serum 0.5 IU/ml})})}$$

Serum sample with a titer lower than 0.5 IU/ml was classified as a negative and a titer ≥ 0.5 IU/ml was a positive.

(A)



(B)

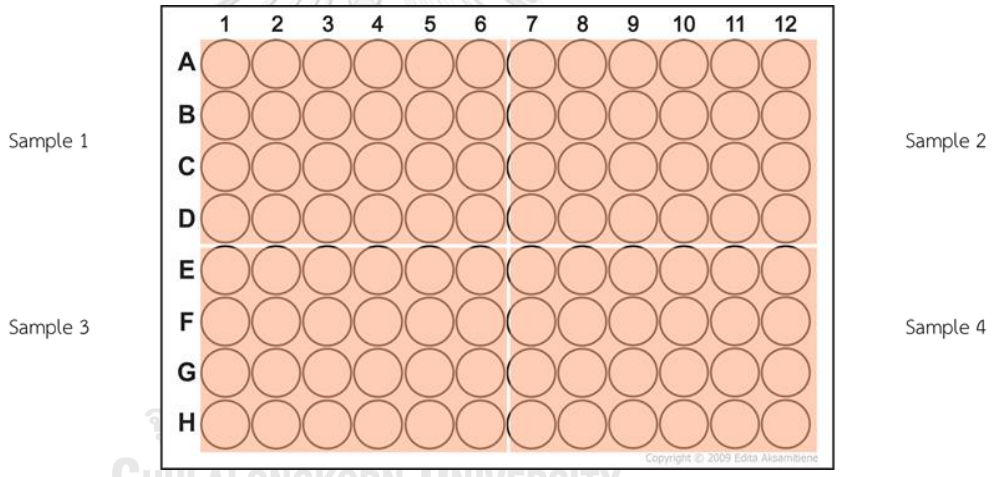


Figure 3 Schematic representation of (A) control plate and (B) test plate for SN-test

Part 4: SN test validation and statistical analysis

4.1 Analytical specificity

Analytical specificity of the SN test was determined by using anti-CDV monoclonal antibody (Creative Diagnostic®, New York, USA).

4.2 Diagnostic sensitivity and specificity

Diagnostic sensitivity was calculated by the formula (true positive / (true positive + false negative) and the diagnostic specificity was calculated following the formula (true negative / (true negative + false positive).

4.3 Repeatability

Repeatability was determined by the coefficient of variation of the E559 monoclonal anti-RABV antibody titer tested on four different dates.

4.4 Correlation of RVNA titer determined by SN-test and FAVN

Correlation between RVNA titers (IU/ml) of 50 dog serum samples by the SN test and FAVN test was calculated by Pearson correlation coefficient method.

CHAPTER 5

Results

Part 1: RABV-pseudotype production

1.1 VSV-pseudotype system

1.1.1 Producer cell and plasmid concentration optimization

As shown in table 10, the pseudotype titers of the HEK293FT producer cell were higher than those of the HEK293T cell. Even though, HEK293FT gave a similar virus titer with both plasmid concentration, the GFP signal of the concentration of 16 ug was brighter than 12 ug. Therefore, HEK293FT was used as a producer cell and transfected with pCAG RABV-G at 16 µg.

Table 10 The titer of VSV(RABV) pseudotype produced by different producer cells and plasmid concentrations

Producer cell	Plasmid concentration (µg)	Titer* ($TCID_{50}$/ml)
HEK293T	12	5.14×10^3
	16	8.63×10^3
HEK293FT	12	1.72×10^4
	16	1.72×10^4

*titration was performed in Vero E6

1.1.2 MOI, time, and target cell

Overall, Vero E6 was slightly more susceptible to the VSV(RABV) pseudotype than BHK-21 at any MOI and production time (Table 11). As shown in Table 11, the VSV(RABV) pseudotype collected at 24 hours post-infection with a recombinant VSVΔG-GFP infection at MOI of 3 gave the highest titer. Therefore, the optimal condition for VSV(RABV) pseudotype production was by using the HEK293FT as a

producer cell with pCAG RABV-G at 16 μg, a recombinant VSVΔG-GFP infection at MOI of 3, a production time of 24 hours and Vero E6 as a target cell.

Table 11 The VSV(RABV) pseudotype titer produced by using different MOI, time and target cells

MOI	Time (hr.)	Target cell	Titer ($TCID_{50}$/ml)
MOI = 1	24	BHK-21	1.89×10^2
	48		3.77×10^2
MOI = 3	24		1.09×10^3
	48		2.24×10^2
MOI = 1	24	Vero E6	9.13×10^2
	48		6.47×10^2
MOI = 3	24		1.82×10^3
	48		1.09×10^3

1.2 Lentiviral pseudotype system

As shown in figure 4, the transfection efficiency of lentiviral vectors was more than 80%. The RABV-lentiviral pseudotype titer was then determined on BHK-21, HEK293T, and Vero E6. As shown in table 12, BHK-21 and HEK293T were slightly more susceptible to the RABV-lentiviral pseudotype than Vero E6.

(A) **(B)**

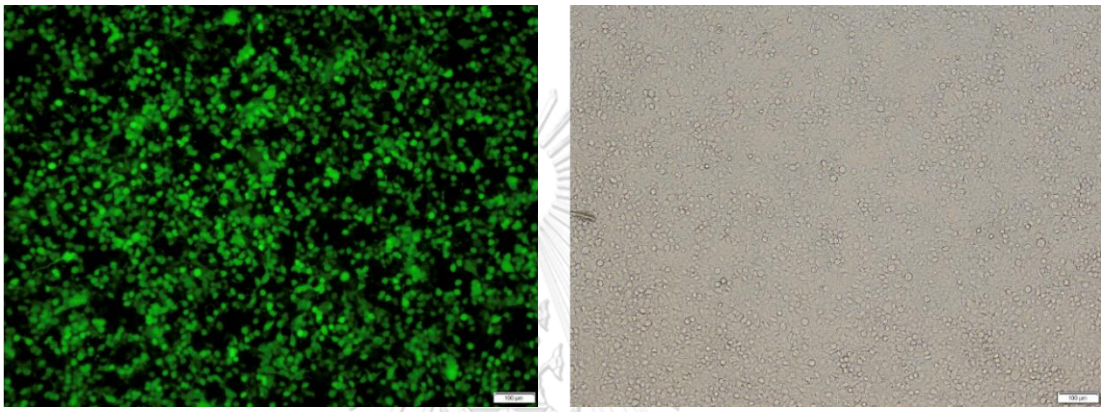


Figure 4 HEK293T cell (producer cells) under the inverted fluorescence microscope at 48 hours post-transfection. (A) GFP field (B) bright field, 100 µm

Table 12 RABV-lentiviral pseudotype titer on different cell lines

Target cell	Titer ($TCID_{50}$/ml)
BHK-21	6.29×10^3
HEK293T	6.29×10^3
Vero E6	1.26 10^3

1.3 RABV-pseudodotype titer comparison

As the titer of the RABV pseudotype produced by the lentivirus system was higher than the VSV system (6.29×10^3 $TCID_{50}$/ml vs. 1.82×10^3 $TCID_{50}$/ml) (Table 13), the lentivirus system was chosen for further study.

Table 13 Comparison of VSV(RABV) pseudotype and RABV-lentiviral pseudotype titer on different target cell

Viral core system	Target cell	Titer ($TCID_{50}$/ml)
VSV(RABV) pseudotype	Vero E6	1.82×10^3
RABV-lentiviral pseudotype	BHK-21	6.29×10^3
	HEK293T	6.29×10^3

Part 2: SN-test protocol optimization

2.1 Infection method and target cell selection

As shown in figure 5, the green fluorescent signal was significantly stronger when target cell was infected by co-culture method. In addition, since co-culture method does not require plating target cells in advance, it can shorten test turnaround time by one day. Regarding the target cell selection, HEK293T was favorable as it gave brighter green fluorescent signal than BHK21 (Figure 5). Moreover the RVNA titers measured on both cells were comparably related with the FAVN titer (Table 14). Therefore, co-culture method and HEK293T were selected as an optimal condition for RABV-lentiviral pseudotype based SN test.

Table 14 Comparison of RVNA titer from different infection method and target cell

Sample No.	Adsorption		Co-culture		FAVN titer (IU/ml)
	BHK21	HEK93T	BHK21	HEK293T	
E559	250	1690	755	737.80	ND*
No.4	0.019	0.17	0.06	0.17	<0.10
No.7	5.75	29.51	10	29.60	>30.77
No.13	1.70	5.00	1.70	2.18	4.50
No.15	1.70	5.00	5.00	5.00	3.42
Correlation**	r = 0.9487, p = 0.1667	r = 0.9487, p = 0.1667	r = 0.8, p = 0.33	r = 0.8, p = 0.33	ND

***ND not determine, **Spearman r test against FAVN titer**

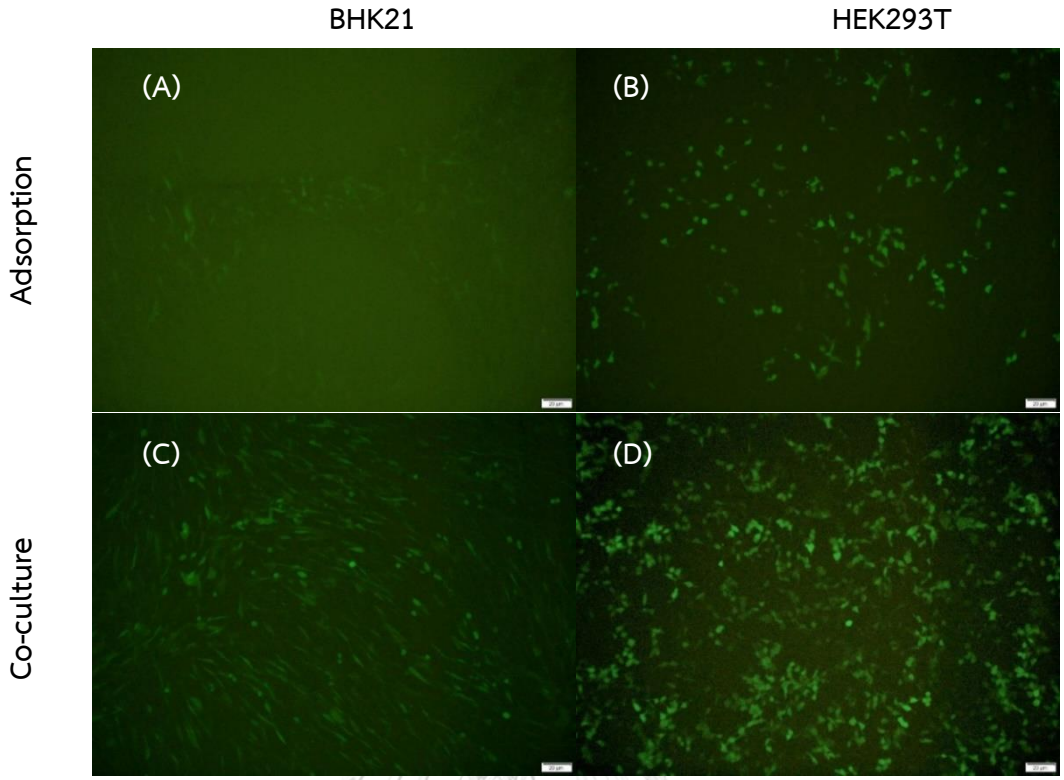


Figure 5 The green fluorescent signal at 48 hours after incubation. (A) Adsorption method in BHK-21, (B) Adsorption method in HEK293T, (C) Co-culture method in BHK-21, (D) Co-culture method in HEK293T, 20 μm

2.2 Cut-off determination

According to the formula I, the RVNA titer is a comparison of neutralization activity observed between unknown serum sample and the standard OIE serum with a known titer of 0.5 IU/ml. Hence, setting a test cut-off of complete reduction (IC100) or 90% reduction in signal (IC90) did not affect the RVNA titer conversion as shown in Table 15. Therefore, the cut-off of IC100 was selected as it provided a clearer demarcation between positive and negative neutralization activity (Figure 6).

Table 15 Comparison of RVNA titer between IC100 and IC90

Sample No.	RVNA titer (IU/ml)		FAVN titer (IU/ml)
	IC100	IC90	
E559	737.8	754.99	ND*
No.4	0.17	0.06	<0.10
No.7	29.6	30.20	>30.77
No.13	2.18	1.70	4.50
No.15	5.00	5.00	3.42
Correlation**	r = 0.8, p = 0.33	r = 0.8, p = 0.33	ND

***ND not determine, **Spearman r test against FAVN titer**

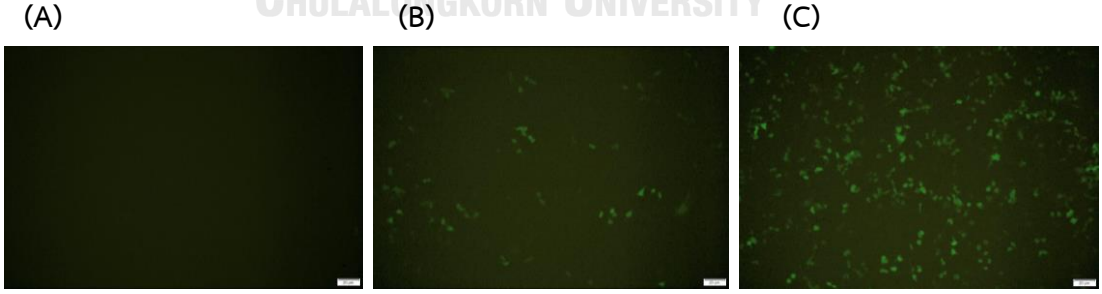


Figure 6 The green fluorescent signal observed in well with (A) complete reduction (IC100), (B) 90% reduction (IC90) and (C) negative serum control, 20 µm

Part 3: Quantification of RVNA titer by SN test

Table 16 summarized the RVNA titer as quantified by the RABV-lentiviral pseudotype-based SN test in comparison with the FAVN titer. From the 50 serum samples, 23 tested positive and 27 tested negative on the pseudotype-based SN test. Whereas 25 sera tested positive and 25 tested negative by the gold standard FAVN test.

Table 16 Comparison of RVNA titer between the RABV-lentiviral pseudotype-based SN test and FAVN test

Sample No.	Code	SN titer (IU/ml)	SN result	FAVN titer (IU/ml)	FAVN result
1	114308/61/26	0.06	-	0.25	-
2	114308/61/30	0.06	-	0.44	-
3	114792/61/3	0.06	-	0.29	-
4	114792/61/27	0.17	-	<0.10	-
5	115922/61/24	0.06	-	0.29	-
6	116092/61/4	4.56	+	>30.77	+
7	116092/61/7	29.6	+	>30.77	+
8	116092/61/10	1.51	+	3.42	+
9	116092/61/13	9.12	+	13.50	+
10	116092/61/25	0.06	-	0.17	-
11	116092/61/29	1.00	+	2.60	+
12	116092/61/37	1.00	+	2.60	+
13	120530/61/1	2.18	+	4.50	+
14	120530/61/2	2.30	+	3.42	+
15	120530/61/3	5.00	+	3.42	+
16	120530/61/4	0.50	+	0.87	+
17	120530/61/5	2.30	+	3.42	+
18	120530/61/8	1.00	+	0.66	+
19	120530/61/11	2.30	+	4.5	+

20	120530/61/12	0.76	+	0.87	+
21	120530/61/14	1.32	+	1.97	+
22	120530/61/16	2.96	+	2.60	+
23	120530/61/19	1.0	+	0.87	+
24	120530/61/22	8.91	+	5.92	+
25	120530/61/26	0.33	-	0.17	-
26	120530/61/27	0.33	-	0.22	-
27	120530/61/28	0.33	-	0.17	-
28	130001/61/2	1.0	+	0.66	+
29	130001/61/5	1.0	+	0.87	+
30	130001/61/6	0.33	-	0.29	-
31	130001/61/13	0.33	-	0.22	-
32	130001/61/14	0.06	-	<0.05	-
33	130001/61/15	4.56	+	13.50	+
34	138123/61/11	0.06	-	<0.02	-
35	138123/61/12	0.06	-	<0.02	-
36	138123/61/13	0.06	-	0.07	-
37	138123/61/14	0.06	-	0.22	-
38	138123/61/15	0.34	-	0.10	-
39	138123/61/16	0.06	-	0.29	-
40	115005/62/1	0.34	-	0.17	-
41	115005/62/2	0.06	-	0.22	-
42	115005/62/3	0.06	-	0.17	-
43	115005/62/4	0.5	+	0.66	+
44	115005/62/5	1.00	+	1.97	+
45	115005/62/6	0.34	-	0.50	+
46	115005/62/7	0.06	-	0.13	-
47	115005/62/8	0.06	-	0.38	-
48	115005/62/9	0.17	-	1.14	+
49	115005/62/11	0.06	-	0.04	-

50	115005/62/14	0.06	-	0.03	-
Positive (+)			23/50		25/50
Negative (-)			27/50		25/50
Total			50/50		50/50

Part 4: SN-test validation and statistical analysis

4.1 Analytical specificity

As shown in figure 7, anti-CDV antibody did not neutralize the RABV-lentiviral pseudotype. This result confirmed the specificity of the RABV-lentiviral pseudotype.

(A) **(B)**

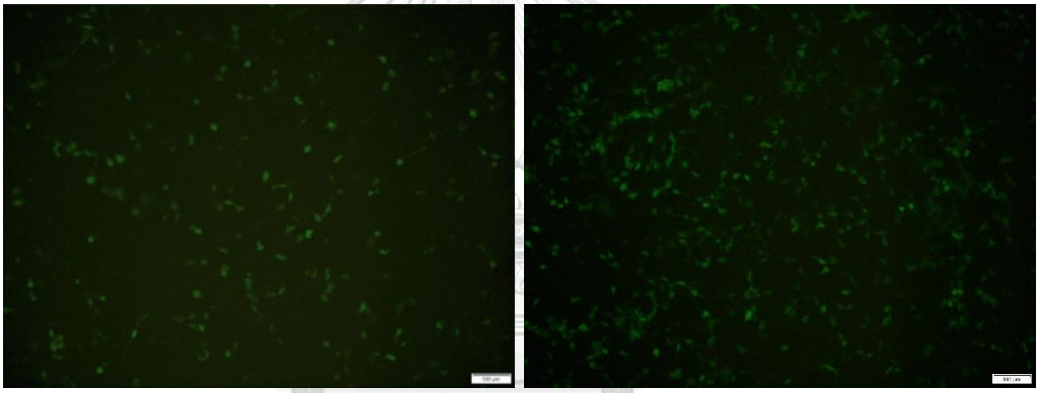


Figure 7 No cross-neutralization of RABV-lentiviral pseudotype by anti-CDV antibody (A) anti-CDV monoclonal antibody, (B) negative serum control, 100 µm

4.2 Diagnostic sensitivity and specificity

From the 50 serum samples, 23 tested positive and 27 tested negative on the RABV-lentiviral pseudotype-based SN test. As there were 2 samples tested positive on FAVN but tested negative on SN test (115005/62/6 and 115005/62/9), the sensitivity of the SN test was 92% (95% CI = 75.03% to 98.58%) with a 100% specificity. (95% CI = 86.88% to 100%) (Fischer's exact test).

Table 17 The RABV neutralizing antibody results by using FAVN test and SN-test

	FAVN Positive	FAVN Negative	Total
SN Positive	23	0	23
SN Negative	2	25	27
Total	25	25	50

Sensitivity

Sensitivity = true positive / (true positive + false negative)

= 23 / 23 + 2

= 0.92

Specificity

Specificity = true negative / (true negative + false positive)

= 25 / 25 + 0

= 1

4.3 Repeatability

As shown on table 13, the RVNA titer of anti-RABV monoclonal antibody, clone E559 tested at 4 different timepoints were consistent, with the coefficient of variation of 1.33. This result confirmed the high repeatability of the RABV-lentiviral pseudotype-based SN test.

Table 18 RVNA titer of the anti-RABV monoclonal antibody, clone E559 tested at different timepoint

	Test date				CV (%)
	26th August 2021	2nd September 2021	13th September 2021	14th September 2021	
titer (IU/ml)	737.8	737.8	755	755	1.33

4.4 Correlation between the RABV-lentiviral pseudotype-based SN test and FAVN

As shown in figure 8, the RVNA titer measured by the RABV-lentiviral pseudotype-based SN test and FAVN test was in a strong positive correlation (Pearson r = 0.9491, p < 0.0001).

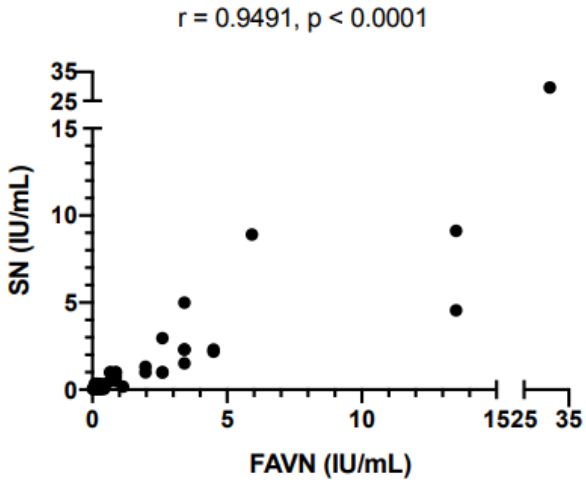


Figure 8 Correlation between the RABV-lentiviral pseudotype-based SN test and FAVN

CHAPTER 6

Discussion

In addition to vaccine coverage, vaccine-induced protection as demonstrated by rabies virus neutralizing antibody (RVNA) titer higher than 0.5 IU/ml is another crucial attribute for the success of Thailand rabies eradication program (Realegeno et al., 2018). However, this parameter is rarely assessed as it requires the use of live rabies virus, which limits the number of testing facility and raises biosecurity concern. Hence, an alternative serological method that could replace the use of live virus was developed in this study. Pseudotyping is a reverse genetic technology that allow expression of envelope glycoproteins of one virus on the core of another. In addition, by replacing glycoprotein gene of the core virus with reporter gene such as green fluorescent protein, the resulting pseudotype becomes replicative incompetent and considered safe.

As different viral backbones could affect pseudotype yield (Li et al., 2018), a RABV pseudotype expressing the glycoprotein of RABV strain CVS-11 based on the 2 most commonly used viral core namely, vesicular stomatitis virus (VSV) and lentivirus, were explored in this study. Our results demonstrated that the titer of lentivirus-based RABV pseudotype was higher titer than the VSV-based pseudotype. This was unexpected as VSV and RABV are both rhabdoviruses with similar replication cycle. The lower titer observed with VSV system is probably due to the highly cytopathic nature of VSV. As demonstrated in this study, HEK293T, a producer cell for pseudotype production, is also susceptible to lentivirus-based RABV pseudotype infection in addition to Vero E6 and BHK-21, the two most commonly used cell lines for RABV infection (Wright et al., 2008). Therefore, it is possible that the newly budded RABV-pseudotype could re-infect the HEK293T cell and induced massive apoptosis (Baxt and Bablanian, 1976). This speculation might also explain a slightly higher titer observed when RABV(VSV) pseudotype was harvested at 24 hours comparing to at 48 hours post-infection. To our surprise, it was demonstrated here

co-culture method is not only possible for the RABV lentiviral pseudotype infection but also resulting in a stronger GFP signal in both HEK293T and BHK-21 cells. It is possible that a larger cell surface area exposed to the RABV-pseudotype in the cell suspension allows a higher level of multiplicity of infection than the cell monolayer in adsorption method.

In Thailand, rabies virus vaccine is usually given together with canine adenovirus type 1 (CAV-1), canine parvovirus (CPV) and canine distemper virus (CDV) vaccines (reference VPAT core vaccine program). Analytical specificity of the RABV pseudotype-based serum neutralization (SN) test developed in this study was demonstrated as it did not cross-neutralize by anti-CDV monoclonal antibody. Although the anti-CPV and anti-CAV-1 antibodies were not available in the market during the time of this study, further validation against these antibodies should be performed to confirm the specificity of the test. Diagnostic sensitivity (92%) and specificity (100%) of the RABV pseudotype-based SN test were high in comparison with the fluorescent antibody virus neutralization (FAVN), a gold standard approved by the World Health Organization (WHO) and Office International des Epizooties (OIE). Of the 50 serum samples tested, 2 samples tested negative by the SN-test but positive by FAVN (No.45, 115005/62/6 and No.48, 115005/62/9). Loss of RVNA titer during the freeze-thawing process might be one of the possible explanations for the observed discrepancy, at least for the sample no.45 as the RVNA titer was already borderline at 0.5 IU/ml by FAVN. Another explanation is poor sample quality as they were collected between 2018-2019. Nevertheless, the overall RVNA titers quantified by the RABV pseudotype-based SN test were comparable with FAVN test. In addition, test repeatability was also demonstrated with the coefficient of variation of only 1.33 among 4 different testing dates.

In addition to our study, RABV SN tests based on other pseudotype systems were reported before. In one study, a RABV-pseudotype based on murine-leukemia virus (MLV) core with green fluorescent (GFP) reporter gene, showed 78.79% sensitivity and 84.62% specificity as compared with gold standard FAVN (Meza et al., 2021). Another study based on lentiviral system and luciferase reporter gene, impressively reported a 100% specificity and sensitivity as compared with the FAVN

assay (Wright et al., 2008). Altogether, results from these studies and ours strongly suggest that the RABV pseudotype-based SN test is a promising alternative method for RVNA quantification.

CHAPTER 7

Conclusion

In summary, the RABV-pseudotype was successfully produced and developed into a quantitative assay for RVNA detection. This new RABV-pseudotype based assay is safer and can be performed in biosafety level 2 facility and with faster turnaround time. The RVNA titer quantified by this new test was comparable to the FAVN, an OIE approved gold standard. Therefore, this newly developed RABV pseudotype-based serum neutralization test is a promising alternative tool for assessing the immune status of the dog population.

REFERENCES

Albertini AA, Ruigrok RW and Blondel D 2011. Rabies virus transcription and replication. Adv Virus Res. 79: 1-22.

Baxt B and Bablanian R 1976. Mechanisms of vesicular stomatitis virus-induced cytopathic effects. I. Early morphologic changes induced by infectious and defective-interfering particles. Virology. 72(2): 370-382.

Benjathummarak S, Fa-Ngoen C, Pipattanaboon C, Boonha K, Ramasoota P and Pitaksajjakul P 2016. Molecular genetic characterization of rabies virus glycoprotein gene sequences from rabid dogs in Bangkok and neighboring provinces in Thailand, 2013-2014. Arch Virol. 161(5): 1261-1271.

Burgado J, Greenberg L, Niezgoda M, Kumar A, Olson V, Wu X and Satheshkumar PS 2018. A high throughput neutralization test based on GFP expression by recombinant rabies virus. PLoS Negl Trop Dis. 12(12): e0007011.

Cliquet F, Aubert M and Sagné L 1998. Development of a fluorescent antibody virus neutralisation test (FAVN test) for the quantitation of rabies-neutralising antibody. J Immunol Methods. 212(1): 79-87.

De Benedictis P, Mancin M, Cattoli G, Capua I and Terrregino C 2012. Serological methods used for rabies post vaccination surveys: an analysis. Vaccine. 30(38): 5611-5615.

Denduangboripant J, Wacharapluesadee S, Lumlertdacha B, Ruankaew N, Hoonsuwan W, Puanghat A and Hemachudha T 2005. Transmission dynamics of rabies virus in Thailand: implications for disease control. BMC Infect Dis. 5: 52.

Fisher CR, Streicker DG and Schnell MJ 2018. The spread and evolution of rabies virus: conquering new frontiers. Nat Rev Microbiol. 16(4): 241-255.

Fodor E 2020. Insight into the multifunctional RNA synthesis machine of rabies virus. Proc Natl Acad Sci U S A. 117(8): 3895-3897.

Fooks AR, Banyard AC, Horton DL, Johnson N, McElhinney LM and Jackson AC 2014. Current status of rabies and prospects for elimination. Lancet. 384(9951): 1389-1399.

Fooks AR, Cliquet F, Finke S, Freuling C, Hemachudha T, Mani RS, Muller T, Nadin-Davis S, Picard-Meyer E, Wilde H and Banyard AC 2017. Rabies. Nat Rev Dis Primers. 3: 17091.

Johnson N, Cunningham AF and Fooks AR 2010. The immune response to rabies virus infection and vaccination. Vaccine. 28(23): 3896-3901.

Kärber G 1931. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Naunyn-Schmiedebergs Archiv für experimentelle Pathologie und Pharmakologie. 162(4): 480-483.

Kasempimolporn S, Saengseesom W, Huadsakul S, Boonchang S and Sitprija V 2011. Evaluation of a rapid immunochromatographic test strip for detection of Rabies virus in dog saliva samples. J Vet Diagn Invest. 23(6): 1197-1201.

Kasempimolporn S, Sichanasai B, Saengseesom W, Puempumpanich S, Chatraporn S and Sitprija V 2007. Prevalence of rabies virus infection and rabies antibody in stray dogs: a survey in Bangkok, Thailand. Prev Vet Med. 78(3-4): 325-332.

Komol P, Sommanosak S, Jaroensrisuwat P, Wiratsudakul A and Leelahapongsathon K 2020. The spread of rabies among dogs in Pranburi district, Thailand: a metapopulation modeling approach. Frontiers in Veterinary Science. 7(925).

Li Q, Liu Q, Huang W, Li X and Wang Y 2018. Current status on the development of pseudoviruses for enveloped viruses. Rev Med Virol. 28(1).

Meza DK, Broos A, Becker DJ, Behdenna A, Willett BJ, Viana M and Streicker DG 2021. Predicting the presence and titre of rabies virus-neutralizing antibodies from low-volume serum samples in low-containment facilities. Transbound Emerg Dis. 68(3): 1564-1576.

OIE 2018a. Rabies. Vol 37 (2). In: Scientific and Technical Review. World Organisation for Animal Health (OIE)

OIE 2018b. Rabies (infection with rabies virus and the other lyssaviruses). In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals World Organization for Animal Health (OIE)

Rampersad S and Tennant P 2018. Replication and Expression Strategies of Viruses. Viruses. 55-82.

Realegeno S, Niezgoda M, Yager PA, Kumar A, Hoque L, Orciari L, Sambhara S, Olson VA

and Satheshkumar PS 2018. An ELISA-based method for detection of rabies virus nucleoprotein-specific antibodies in human antemortem samples. PLoS One. 13(11): e0207009.

Servat A, Feyssaguet M, Blanchard I, Morize JL, Schereffer JL, Boue F and Cliquet F 2007. A quantitative indirect ELISA to monitor the effectiveness of rabies vaccination in domestic and wild carnivores. J Immunol Methods. 318(1-2): 1-10.

Smith JS, Yager PA and Baer GM 1973. A rapid reproducible test for determining rabies neutralizing antibody. Bull World Health Organ. 48(5): 535-541.

Spearman C 1908. The method of 'right and wrong cases' ('constant stimuli') without Gauss's formulae. British Journal of Psychology, 1904-1920. 2(3): 227-242.

Toon K, Bentley EM and Mattiuzzo G 2021. More than just gene therapy vectors: Lentiviral vector pseudotypes for serological investigation. Viruses. 13(2).

Wasniewski M and Cliquet F 2012. Evaluation of ELISA for detection of rabies antibodies in domestic carnivores. J Virol Methods. 179(1): 166-175.

Weir DL, Annand EJ, Reid PA and Broder CC 2014. Recent observations on Australian bat lyssavirus tropism and viral entry. Viruses. 6(2): 909-926.

Whitt MA 2010. Generation of VSV pseudotypes using recombinant DeltaG-VSV for studies on virus entry, identification of entry inhibitors, and immune responses to vaccines. J Virol Methods. 169(2): 365-374.

WHO 2005. WHO Expert Consultation on Rabies : first report. In: WHO technical report series; 931. World Health Organization, Geneva.

World Health O 2018. Rabies vaccines: WHO position paper, April 2018 - Recommendations. Vaccine. 36(37): 5500-5503.

Wright E, Temperton NJ, Marston DA, McElhinney LM, Fooks AR and Weiss RA 2008. Investigating antibody neutralization of lyssaviruses using lentiviral pseudotypes: a cross-species comparison. J Gen Virol. 89(Pt 9): 2204-2213.

VITA

NAME	Nichapat Rojjananavin
DATE OF BIRTH	January 8, 1995
PLACE OF BIRTH	Thailand
INSTITUTIONS ATTENDED	Bachelor's degree from Faculty of Veterinary Technology with second-class honours, Kasetsart University in 2017
PUBLICATION	Rojjananavin N, Kaewkhunjob E, Molee L, Thonghem J, Songkasupa T, Wandee N, Techakriengkrai N 2020. Development of a replicative incompetent rabies virus (RABV) pseudotype based on a recombinant vesicular stomatitis virus (VSV) particle. Proceeding of The 19th Chulalongkorn University Veterinary Conference CUVC2020, Bangkok, Thailand 18-19 October 2020: 86-87. Tangjirawattana C, Kaewkhunjob E, Rojjananavin N, Techakriengkrai N 2020. Felis catus gammaherpesvirus 1 (FcaGHV1) in domestic cats in Bangkok, Thailand. Proceeding of The 19th Chulalongkorn University Veterinary Conference CUVC2020, Bangkok, Thailand 18-19 October 2020: 102-103. Kaewkhunjob E, Rojjananavin N, Bodgener J, Gilbert M, Techakriengkrai N 2020. A retrospective study of canine distemper virus seroprevalence in captive wildlife in Thailand. Proceeding of The 19th Chulalongkorn University Veterinary Conference CUVC2020, Bangkok, Thailand 18-19 October 2020: 347-348.
AWARD RECEIVED	Second Prize Oral Presentation Award of The 19th