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Molecular detection and genetic diversity of rotavirus a in domestic dogs and cats in Bangkok, Thailand

Ekkapat Chamsai
Faculty of Veterinary Science

MOLECULAR DETECTION AND GENETIC DIVERSITY OF ROTAVIRUS A IN DOMESTIC DOGS AND CATS IN BANGKOK, THAILAND

Mr. Ekkapat Chamsai

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การตรวจพิสูจน์และความหลากหลายทางพันธุกรรมของโรตาไวรัสเอในสุนัขและแมว ในจังหวัดกรุงเทพมหานคร ประเทศไทย

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Thesis Advisor	Professor ALONGKORN AMONSIN, D.V.M., Ph.D.

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

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

THESIS COMMITTEE

..... Chairman

(Professor RUNGTIP CHUANCHUEN, D.V.M., M.S., Ph.D.)

..... Thesis Advisor

(Professor ALONGKORN AMONSIN, D.V.M., Ph.D.)

..... Examiner

(Assistant Professor TARADON LUANGTONGKUM, D.V.M., Ph.D.)

..... Examiner

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

..... External Examiner

(Assistant Professor KAMOL SUWANNAKARN, Ph.D.)

เอกพัทธ์ แจ่มใส : การตรวจพิสูจน์และความหลากหลายทางพันธุกรรมของโรตาไวรัสเอในสุนัขและแมว ในจังหวัดกรุงเทพมหานคร ประเทศไทย. (MOLECULAR DETECTION AND GENETIC DIVERSITY OF ROTAVIRUS A IN DOMESTIC DOGS AND CATS IN BANGKOK, THAILAND) อ.ที่ปรึกษาหลัก : ศ. น.สพ.ดร.อลงกร อมรศิลป์

เชื้อโรตาไวรัสเอ เป็นเชื้อก่อโรคที่มีความสำคัญและก่อให้เกิดกลุ่มอาการทางเดินอาหารอักเสบในคนและสัตว์หลายชนิด เนื่องจากเชื้อโรตาไวรัสเอมีความสามารถในการติดเชื้อจากสัตว์สู่คนได้ จึงมีความสำคัญและควรเฝ้าระวัง โดยเฉพาะในสัตว์ที่มีความใกล้ชิดกับคน เช่น สุนัขและแมว เป็นต้น การศึกษานี้มีวัตถุประสงค์เพื่อหาอุบัติการณ์ของเชื้อโรตาไวรัสเอในสุนัขและแมว รวมถึงหาความหลากหลายทางพันธุกรรม และความสัมพันธ์ทางพันธุกรรมของเชื้อโรตาไวรัสเอ ในสุนัข แมว และสัตว์ชนิดต่างๆ รวมทั้งคน การศึกษานี้ได้เก็บตัวอย่างป้ายทวารหนักจากสุนัขและแมว ในโรงพยาบาลสัตว์ ในเขตกรุงเทพมหานครและปริมณฑล ระหว่างเดือนมกราคม พ.ศ.2563 ถึงเดือนมิถุนายน พ.ศ.2564 รวมทั้งหมด 572 ตัวอย่าง ผลการตรวจพิสูจน์หาเชื้อโรตาไวรัสเอ ในสุนัขและแมว ด้วยวิธี conventional one-step RT-PCR พบอุบัติการณ์ของเชื้อไวรัส 1.92% (11/572) ในสุนัขและแมว โดยแบ่งเป็น 2.75% (8/290) ในสุนัข และ 1.06% (3/282) ในแมว โดยอายุอาจเป็นปัจจัยเสี่ยงที่สำคัญต่อการติดเชื้อโรตาไวรัสเอในสุนัข แต่ผลของปัจจัยเสี่ยงในแมวยังไม่ชัดเจน นอกจากนี้การพบเชื้อโรตาไวรัสเอในแมวเป็นรายงานการพบเชื้อไวรัสเป็นครั้งแรกในประเทศไทย การศึกษานี้ได้ถอดรหัสพันธุกรรมทั้งหมดของเชื้อโรตาไวรัสเอ จากสุนัขจำนวน 2 ตัวอย่าง และเชื้อโรตาไวรัสเอ จากแมวจำนวน 1 ตัวอย่าง ด้วยวิธี nanopore sequencing โดยผลการถอดรหัสพันธุกรรมพบว่า เชื้อโรตาไวรัสเอทั้งหมดจัดอยู่ในจีโนไทป์ G3P[3] โดยรหัสพันธุกรรมทั้งหมดของเชื้อโรตาไวรัสเอในสุนัขมีรูปแบบจีโนไทป์ G3-P[3]-I3-R3-C3-M3-A9-N2-T3-E3-H6 ส่วนโรตาไวรัสเอในแมวมีรูปแบบจีโนไทป์ G3-P[3]-I8-R3-C3-M3-A9-N3-T3-E3-H6 และเชื้อโรตาไวรัสเอในสุนัขและแมวนี้ ถูกจัดอยู่ในกลุ่มจีโนไทป์ AU-1 และมีการแลกเปลี่ยนสารพันธุกรรมของเชื้อไวรัสจากกลุ่มจีโนไทป์อื่น ซึ่งจากผลการวิเคราะห์รหัสพันธุกรรมพบว่า เชื้อโรตาไวรัสเอในสุนัขมีพันธุกรรมคล้ายกับเชื้อโรตาไวรัสเอในสุนัขจากงานวิจัยล่าสุดในประเทศไทย ใน 9 ยีนหลัก ยกเว้นยีน VP6 และ NSP3 ที่มีความคล้ายกับเชื้อตาไวรัสเอที่พบในคนและเชื้อโรตาไวรัสเอที่พบในแมว ตามลำดับ และผลการวิเคราะห์ด้วยวิธี bootscan analysis ยังสนับสนุนถึงความเป็นไปได้ของการแลกเปลี่ยนสารพันธุกรรมของเชื้อไวรัสเหล่านี้ ส่วนผลการวิเคราะห์รหัสพันธุกรรมของเชื้อโรตาไวรัสเอในแมว พบว่า เชื้อไวรัสมีความคล้ายกับเชื้อโรตาไวรัสเอในค้างคาว คน และสัตว์ตระกูลลิง และเมื่อวิเคราะห์ด้วยวิธี bootscan analysis ก็ยืนยันถึงความเป็นไปได้ของการเกิดการแลกเปลี่ยนสารพันธุกรรมของเชื้อไวรัสจากไวรัสในสัตว์แต่ละชนิดเหล่านั้น อย่างไรก็ตามเชื้อโรตาไวรัสเอในแมวในการศึกษานี้ไม่มีความใกล้เคียงกับเชื้อโรตาไวรัสเอในแมวจากการศึกษาอื่นๆ จึงอาจสันนิษฐานได้ว่าเชื้อโรตาไวรัสเอที่พบในแมว อาจได้รับการถ่ายทอดมาจากสัตว์ชนิดอื่น และอาจเกิดการแลกเปลี่ยนสารพันธุกรรมของเชื้อไวรัสในสัตว์ชนิดนั้น โดยสรุปการศึกษานี้พบอุบัติการณ์ของเชื้อโรตาไวรัสเอในสุนัขและแมว และพบหลักฐานการเกิดการแลกเปลี่ยนสารพันธุกรรม, การแพร่เชื้อไวรัสจากสัตว์ชนิดหนึ่งไปสู่อีกชนิดหนึ่ง รวมถึงความเป็นไปได้ของการแพร่เชื้อไวรัสจากสัตว์สู่คนด้วย เพราะฉะนั้นจึงควรเพิ่มความตระหนักถึงเชื้อโรตาไวรัสเอในสุนัขและแมวในด้านสาธารณสุข และควรมีการศึกษาเชื้อโรตาไวรัสเอในสุนัขและแมวเพิ่มเติมเป็นวงกว้างขึ้นเพื่อทราบข้อมูลความชุก การแพร่กระจาย และความรุนแรงของเชื้อไวรัส รวมถึงควรมีการศึกษาเฝ้าระวังเพิ่มเติมของเชื้อโรตาไวรัสเอในค้างคาว สัตว์ตระกูลลิง และสัตว์ป่าชนิดอื่นๆ เพื่อวิเคราะห์หาต้นกำเนิดและวิวัฒนาการของเชื้อไวรัสต่อไปในอนาคต

สาขาวิชา	สัตวแพทยสาธารณสุข	ลายมือชื่อนิสิต .....
ปีการศึกษา	2564	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Ekkapat Chamsai : MOLECULAR DETECTION AND GENETIC DIVERSITY OF ROTAVIRUS A IN DOMESTIC DOGS AND CATS IN BANGKOK, THAILAND. Advisor: Prof. ALONGKORN AMONSIN, D.V.M., Ph.D.

Rotavirus A (RVA) in one of the important pathogens causing gastroenteritis in humans and many animal species. The zoonotic potential of RVA has been reported and raises major concerns, especially in high animal-human interfaces settings. The objectives of this thesis were to determine the occurrence of RVAs in dogs and cats, and to investigate the genetic diversity and genetic relationship among RVAs in dogs, cats, human, and other animal species. The total of 572 rectal swab samples were collected from dogs and cats in animal hospitals, in Bangkok and vicinities, during January 2020 to June 2021. The conventional one-step RT-PCR result showed 1.92% (11/572) occurrence of RVAs in dogs and cats, which by species were 2.75% (8/290) in dogs, and 1.06% (3/282) in cats. Age of animals might be the risk factor affecting the occurrence of RVAs in dogs, but the result in cats is still inconclusive. Besides, our finding is the first report of rotavirus A in cats in Thailand. To characterize the viruses, two canine rotavirus A (CRVA) and one feline rotavirus A (FRVA) were subjected to whole genome sequencing by nanopore sequencing. Our results showed all 3 viruses were classified as RVA genotype G3P[3]. Genetic constellation of RVAs, CRVAs had G3-P[3]-I3-R3-C3-M3-A9-N2-T3-E3-H6 genotype, while FRVA had G3-P[3]-I8-R3-C3-M3-A9-N3-T3-E3-H6 genotype. Notably, RVAs in this study had the AU-1 genetic constellation backbone with reassortment. The results of genetic analysis showed that CRVAs were closely related and had high nucleotide identities to CRVAs from previous reported in Thailand, except VP6 gene and NSP3 gene which were closely related to RVAs in human and cat, respectively. The result of bootscan analysis supported the possible reassortment of RVAs from dog, human, and cat in the CRVAs. While FRVA in this study was closely related and had high nucleotide identities to RVAs in human, bat, and simian. The result of bootscan analysis also supported the possible reassortment of this FRVA. Meanwhile, none of any segments of this FRVA were closely related to cat's reference strain, indicating possible interspecies transmission of FRVA in intermediate animal species to cat, and the reassortment event of RVAs from human, bat, and simian had occurred in that intermediate animal species. In conclusion, this study provided the occurrence of RVAs in dogs and cats, and suggesting the possible multiple reassortment, interspecies transmission, as well as zoonotic potential of the viruses. The public health awareness should be raised due to the zoonotic potential of CRVA and FRVA. RVAs studies in larger scale in dogs and cats in Thailand should be considered to determine the dynamic and distribution of the viruses. In addition, the studies of RVAs in bats, simian, and other wildlife species should be performed to analyze the origin and evolution of RVAs in the future.

Field of Study:	Veterinary Public Health	Student's Signature .....
Academic Year:	2021	Advisor's Signature .....

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Ekkapat Chamsai

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LIST OF ABBREVIATIONS

bp	Base pair(s)
cDNA	Complementary deoxyribonucleic acid
CRVA	Canine rotavirus A
DNA	Deoxyribonucleic acid
EIA	Enzyme immunoassays
et al.	Et alibi, and others
FRVA	Feline rotavirus A
ICTV	International Committee on Taxonomy of Viruses
MEM	Eagle minimum essential medium
min	Minute(s)
mth	Month(s)
NA	Not available
PPE	Personal protective equipment(s)
RT-PCR	Reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
RV	Rotavirus
RVA	Rotavirus A
temp	Temperature
WGS	Whole genome sequencing
yr	Year(s)

°C	Degree Celsius
µg	Microgram(s)
µl	Microliter(s)
µM	Micromolar

Chapter 1
Introduction

1.1 Importance and Rationale

Rotavirus (RV) is a non-enveloped, double stranded RNA virus of the family Reoviridae, genus Rotavirus. The virus consists of 11 RNA segments encoding 6 structural proteins (VP1-VP4, VP6, VP7) and 5 or 6 nonstructural proteins (NSP1-NSP5/NSP6). Up to date, rotavirus can be classified into species A to H with other 2 proposed species I and J (Matthijnssens et al., 2012; Mihalov-Kovács et al., 2015; Banyai et al., 2017). Rotavirus species A (RVA) is the most important species causing gastroenteritis in many animal species as well as human especially in children.

In human, RVA infection causes severe diarrhea in children and can be fatal. Previous studies reported that about 50-60% of hospitalized diarrhea children age under 5 years old were infected with RVA (Parashar et al., 2006; Palumbo et al., 2009; Tate et al., 2012). Despite the use of rotavirus vaccine, there are still outbreaks of gastroenteritis from rotavirus worldwide especially in developing countries. The rotavirus outbreaks could be due to the emerging of novel RVA strains or reassortment of the RVAs from different origins. Some strains of RVAs have been reported as zoonotic potential (Banyai et al., 2010; Grant et al., 2011; Doro et al., 2015). In animals, RVA infection causing gastroenteritis has been reported in many animal species. RVA infection can affect the animal production especially swine and poultry industries (Saikruang et al., 2013; Otto et al., 2015). In dogs and cats, RVA can cause mild diarrhea in puppies and kittens, but mostly subclinical and self-limiting (Martella et al., 2010). It has been reported of inter-species transmission of RVA between dogs and children with severe diarrhea (De Grazia et al., 2007; Wu et al., 2012). Moreover, evidence of multiple reassortments of RVA between feline and human rotaviruses have also been documented (Grant et al., 2011). Since dogs and cats are usually interface with human and rotavirus can transmit by fecal-oral route,

thus RVAs can spread easily and increase the chance of interspecies transmission or viral reassortment which subsequently generate novel strains of RVA with zoonotic potential.

There are several reports of RVA infection in dogs and cats worldwide. For example, some studies reported the evidence of interspecies transmission from dogs and cats to humans as well as the reassortment among RVAs (De Grazia et al., 2007; Grant et al., 2011; Luchs et al., 2012; Matthijnssens and Van Ranst, 2012; Wu et al., 2012; Otto et al., 2015). However, there is limit information of RVA in dogs and cats in Thailand. Thus, this thesis will be conducted to provide the status, distribution, and genetic information of RVA circulating in dogs and cats in Thailand. The information from this thesis will be useful for public on the awareness and risk of RVA infection in persons who have close contact with dogs and cats and will provide recommendations for RVA prevention and control in domestic animals and humans.

1.2 Research questions

1. What is the occurrence and distribution of rotavirus A (RVA) in domestic dogs and cats in Bangkok, Thailand?
2. How diverse of the genetic of rotavirus A (RVA) isolated from dogs and cats? Are there any reassortment among RVAs isolated from dogs and cats?

1.3 Objectives of Study

1. To determine the occurrence of rotavirus A (RVA) in domestic dogs and cats in Bangkok, Thailand.
2. To determine the genetic characteristics and genetic diversity of rotavirus A (RVA) in domestic dogs and cats in Bangkok, Thailand.

Chapter 2
Literature Review

2.1 Virology of Rotavirus

Rotavirus (RV) is an icosahedral, triple-layered, nonenveloped, double-stranded RNA virus of the family Reoviridae, genus Rotavirus. The size of RV is 70–75 nm in diameter. The genome of RV consists of 11 segments of double-stranded RNA that encoded 6 structural proteins (VP1, VP2, VP3, VP4, VP6, VP7) and five or six non-structural proteins (NSP1, NSP2, NSP3, NSP4, NSP5-6) depending on strains (Estes and Cohen, 1989; Pesavento et al., 2006). The classification system of RV is based on the antigenic properties of VP6. RV can be subdivided into eight species A to H and two additional proposed species (I and J) according to the International Committee on Taxonomy of Viruses (ICTV) (Matthijnssens et al., 2012). Rotavirus species A, B, C, and E (RVA, RVB, RVC, and RVE) are known to infect humans and various animal species (Matthijnssens et al., 2011a). Rotavirus species D, F and G (RVD, RVF and RVG) have been recovered from animals, mostly from birds (Matthijnssens et al., 2011a). While rotavirus species H (RVH) mostly found in pigs (Marthaler et al., 2014; Molinari et al., 2014). The latest two proposed rotavirus species I and J (RVI and RVJ) were found in dogs (Mihalov-Kovács et al., 2015) and bats (Banyai et al., 2017), respectively. In present, RVA is known to be the most important species causing gastroenteritis in many animal species as well as human especially in children.

2.2 Genotyping of RVA

Rotavirus A (RVA) can be classified based on two classification system. The first classification system is based on the outer layer protein VP7 and VP4 which designated as genotype Gx[Px]. Another system for RVA classification by the rotavirus classification working group (RCWG) classify the virus based on 11 genes which designated as Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx representing the genetic

constellation of VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 genes respectively, x indicating the number of genotype (Matthijnssens et al., 2008b).

From previous studies, the complete genome of RVA can be divided into 3 genetic constellations. The first constellation is Wa-like (P8-I1-R1-C1-M1-A1-N1-T1-E1-H1) in combination with different G-genotypes, such as G1, G3, G4 and G9), and is revealed as a porcine origin. Second, the constellation defined as DS-1-like (G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2) has been associated with bovine rotaviruses. Both Wa-like and DS-1-like have circulated in humans worldwide (Matthijnssens et al., 2008a; Ghosh and Kobayashi, 2011; Matthijnssens and Van Ranst, 2012). Third genetic constellation is AU-1-like (G3-P[9]-I3-R3-C3-M3-A3-N3-T3-E3-H3) which is believed to have originated from cat or dog viruses (Nakagomi et al., 1990).

2.3 Transmission, Diagnosis, and Epidemiology of RVA

Rotavirus is known to be transmitted person-to-person by the fecal oral route (Butz et al., 1993). In developing countries rotavirus can also be transmitted via fecal contaminated water (Ansari et al., 1991). Rotavirus infection has a short incubation period of 1 to 3 days. The virus can shed for up to 48 hours before person exhibit clinical symptoms. High rates of asymptomatic shedding of rotavirus have been reported in young children. The virus can shed in feces for an average of 4 days and up to 30 days in immunocompromised patients (Dennehy, 2000).

For sample collection method, fecal collection is the gold standard for the detection of RVA but for convenient, the rectal swab is acceptable. There are many methods to detect RVA infection. Enzyme immunoassays (EIA) is one method usually use for the diagnosis of RVA infection and there are many commercial kits available. However, the sensitivity of the assay is low when compared to polymerase chain reaction (PCR) method. There are 28% more RVA detection rate of one-step quantitative RT-PCR (Q-PCR) compare to EIA (Zeng et al., 2008).

Previous study reported that the patterns of RVA infection could occur year-round and more frequent in low and low-middle income countries. While upper-middle and high income countries, RVA infection is more likely to be seasonal. It has been reported that the seasonality of RVA infection relate to levels of country development more than geographic location or climate (Patel et al., 2013). In Thailand, the low-middle income country, the RVA infection rate is always highest during low temperature period around December to February (Khananurak et al., 2010; Chieochansin et al., 2016). The studies in Thailand showed that RVA can be infected in all age groups (2 months to 86 years) but mostly in the children less than 5 years of age. The most affected age is range between 12 months and 17 months (Intusoma et al., 2008; Khananurak et al., 2010; Kittigul et al., 2014).

2.4 RVA infection in humans and animals

In human, the most common clinical signs of RVA infection are severe diarrhea and vomiting, resulting in dehydration. It can also cause nausea, malaise, headache, abdominal cramping, and fever (Anderson and Weber, 2004). RVA infection in human can be varying between 28% and 59% in Asian countries (Nelson et al., 2008). The previous study in Thailand between 2007 to 2009 reported the prevalence of RVA as 28.4% in children with acute gastroenteritis (Khananurak et al., 2010). Another study between 2011 to 2014 reported 30% of RVA infection (Chieochansin et al., 2016). There are predominant genotypes of RVAs in human in Thailand, G1P[8], G2P[4], and G3P[8] (Khananurak et al., 2010). In zoonotic aspect, genotype G9P[8] that is the porcine-like genotype had been found in high percentage (22.2%) between 2007 to 2009 (Khananurak et al., 2010) and also the bovine-like genotype G8P[8] (13%) had been reported between 2011 to 2014 (Chieochansin et al., 2016).

In dogs and cats, RVA infection causing acute gastroenteritis had been reported in many studies (Hoshino et al., 1981; Hoshino et al., 1982; Nakagomi et al., 1989; Mochizuki et al., 1997; Martella et al., 2001a; Martella et al., 2001b; Kang et al., 2007). In dogs, most clinical signs can be observed in pups less than 3 months of age and normally asymptomatic in adult dogs. The clinical signs cause by RVA in dogs are diarrhea, lack of appetite, and lethargy (Hoshino et al., 1982; Martella et al., 2001b; Kang et al., 2007). A previous study in Thailand found 3 out of 5 dogs positive with RVA without clinical signs (Charoenkul et al., 2020). In cats, RVA infection has been reported to cause both symptomatic and asymptomatic, but subclinical infection is more often observed. Mild and transient diarrhea is likely to be seen in kittens, especially in the kitten lack of colostrum (Hoshino et al., 1981). The prevalence of RVA infection in dogs and cats is varying. Previous study reported high prevalence of RVA infection in dogs at 39.7% (31/78) and even more in cats at 50.0% (23/46) (Otto et al., 2015). Another study in Thailand found only 0.7% (5/710) RVA positive with RVA in dogs (Charoenkul et al., 2020). Most studies reported about 2-8% prevalence of RVA infection in dogs (Ortega et al., 2017; Alves et al., 2018). In United Kingdom reported only 3% (57/1727) positive of RVA in cat feces (German et al., 2015). Worldwide, the predominant RVA genotype in dogs is G3P[3] (De Grazia et al., 2007; Tsugawa and Hoshino, 2008; Martella et al., 2010; Matthijnssens et al., 2011b; Doro et al., 2015; Mihalov-Kovács et al., 2015). In cats, G3P[3] and G3P[9] are recognized as feline genotype (AU-1-like and BA222-like genotype constellations) (Nakagomi and Nakagomi, 1989; Nakagomi et al., 1990; Martella et al., 2011). But latest study in United Kingdom reported the first G6P[9] detection in cats (German et al., 2015). In Thailand, there is only report of G3P[3] found in dogs (Charoenkul et al., 2020) but none of report in cats.

In zoonotic aspect, a few studies, genotypes G3P[3], G3P[9], and G6P[9] have been reported in humans in many countries. Genotype G3P[3] had been found in Italy (De Grazia et al., 2007), and Japan (Tsugawa and Hoshino, 2008). Genotype

G3P[9], which is believed to be direct transmission from dogs or cats had been reported in humans in Japan (Nakagomi and Nakagomi, 1989), Israel (Silberstein et al., 1995), Brazil (Santos et al., 2001), Russia (Novikova et al., 2008), and Hungary (Bányai et al., 2009). Genotype G6P[9], which is potential zoonotic or reverse-zoonotic transmission between cats and people (German et al., 2015) had been reported in the United States (Griffin et al., 2002), Hungary (Bányai et al., 2003; Bányai et al., 2009), Japan (Yamamoto et al., 2011), Australia (Cooney et al., 2001; Diwakarla et al., 2002), and Tunisia (Ben Hadj Fredj et al., 2013). In Thailand, genotype G3P[9] in human has been reported between 2007 to 2009 (Khananurak et al., 2010) and 2011 to 2014 (Chieochansin et al., 2016). Another study in Thailand also reported genotype G3P[3] from children in Chiangmai (Khamrin et al., 2006).

The whole genome analysis of RVA in Thailand, RVA strain, G3-P[9]-I3-R3-C3-M3-A3-N3-T3-E3-H6 from human contains AU-1 -like genomic constellation with re-assortment of gene 3 (VP6) and gene 1 1 (NSP5), suggesting possible re-assortment events between human and canine strains (Theamboonlers et al., 2014). The study of whole genome of RVA from dogs in Thailand revealed a genotype constellation G3-P[3]-I3-R3-C3-M3-A9-N2-T3-E3-H6 which is the novel constellation in dogs but had been reported in human before. This constellation belongs to the genotype AU-1-like with combination of 10 segments in Cat-like genogroup. This result can be speculated that RVA in dogs in Thailand could have originated from multiple reassortment or intra-genotype reassortment between human and animal RVAs (Charoenkul et al., 2020). Due to the zoonotic potential of rotaviruses, routine surveillance of rotaviruses will be useful for monitoring the emergence of novel strains of rotaviruses through interspecies reassortment between human and animal viruses.

Chapter 3
Materials and methods

This thesis composes of three study phases including Phase 1) Sample collection from dogs and cats in animal hospitals in Bangkok and the vicinities. Phase 2) Identification of rotavirus A (RVA) in dogs and cats by specific RT-PCR assay. Phase 3) Genetic characterization of rotavirus A (RVA) in dogs and cats by whole genome sequencing.

The conceptual framework of this study is shown in Figure 1.

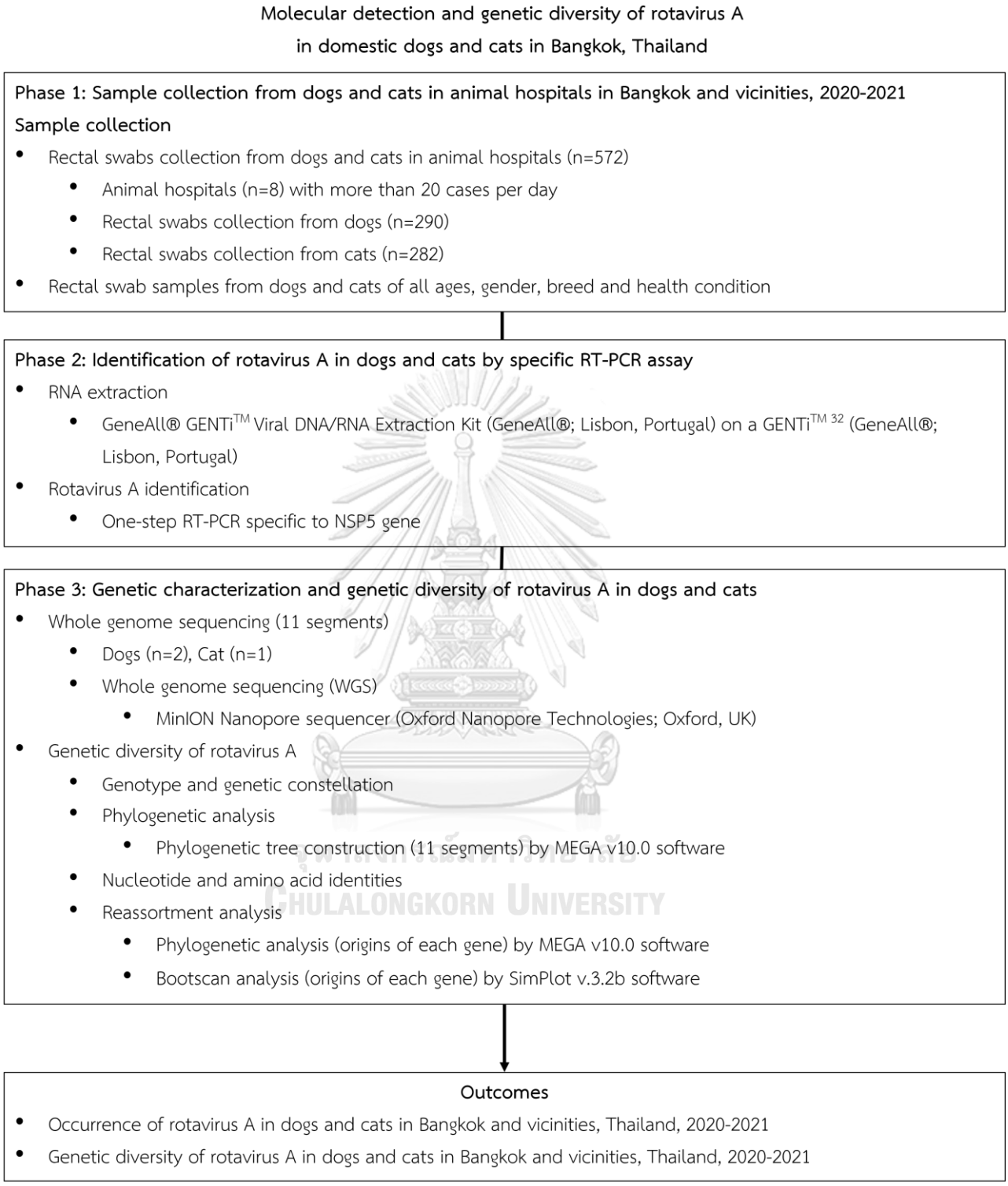


Figure 1: Conceptual framework of this study

3.1 Phase 1: Sample collection from dogs and cats in animal hospitals in Bangkok and the vicinities

3.1.1 Study sites

In this thesis, samples were collected from animal hospitals located in Bangkok and Nonthaburi provinces. In total 8 animal hospitals were selected and included in this thesis. The animal hospitals were selected by 1) scale of the animal hospital in which average of 20 or more cases per day and 2) cooperation of owners, veterinarians, and healthcare workers. The locations of the animal hospitals are shown in Figure 2.

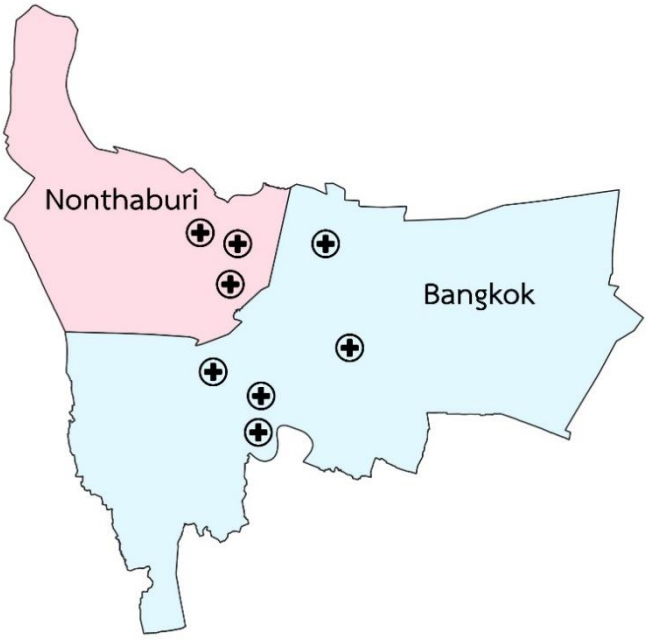


Figure 2: Location of animal hospitals in Bangkok and Nonthaburi included in this study.

⊕ Symbol indicate location of the animal hospitals. Blue and pink zones zone represent Bangkok and Nonthaburi, respectively.

3.1.2 Sample collection from dogs and cats

Rectal swab samples were collected from dogs and cats of all ages, gender, breed, and health condition that come to have service at the animal hospitals

(Figure 3). Rectal swab sampling was conducted by insert the cotton swab into rectum and rotate gently (Figure 4 and Figure 5). The swabs were placed in viral transport media (MEM; eagle minimum essential medium) and kept at 4°C and transported to laboratory within 24 hours.

In this thesis, the sample collection was carried out from January 2020 to June 2021 (18 months). The total of 572 rectal swabs were collected from dogs (n = 290) and cats (n = 282). The animal's demographic data, including age, sex and health condition were also recorded.

Figure 3: Rectal swab sample collection at animal hospital

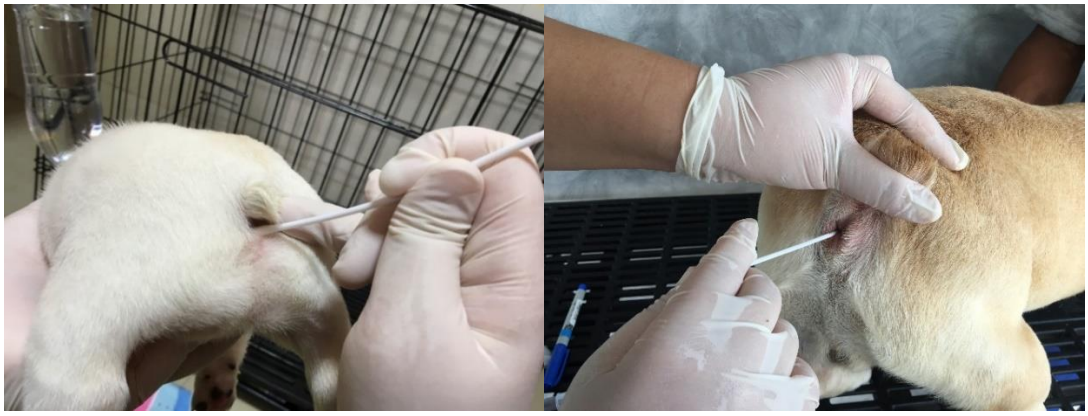


Figure 4 and Figure 5: Rectal swab procedure

3.1.3 Sample preparation

Rectal swab samples were aliquoted into 200ul for RNA extraction. RNA extraction was processed by using GeneAll® GENTi™ Viral DNA/RNA Extraction Kit

(GeneAll®; Lisbon, Portugal) on a GENTi™ 32 (GeneAll®; Lisbon, Portugal) following manufacturer's instruction. In brief, first, 200µl of aliquoted samples were put in the first channel of GeneAll® GENTi™ Viral DNA/RNA Extraction Kit (GeneAll®; Lisbon, Portugal) to mix with the lysis buffer. The 7ul of RNA carrier was then added to the first channel of the kit to mix with the sample. The extraction kit was then put in GENTi™ 32 extraction machine (GeneAll®; Lisbon, Portugal) for further process, the magnetic beads in the kit were activated by the machine to extract the RNA from the sample and then eluted by the elution buffer. The extracted RNA was kept at -20°C until virus identification.

3.2 Phase 2: Identification of RVA in dogs and cats by specific RT-PCR assay

The RNA samples were subjected to rotavirus A (RVA) identification by one-step RT-PCR using specific primer to the conserve region of NSP5 gene of canine and feline rotavirus. The newly designed primers used in the RT-PCR assay were NSP5F (5'-ACAACGTCAACTCTTTCTGGA-3') and NSP5R (5'-GATGAATCCATAGACACGCC-3') which designed by Primer 3 plus program. The one-step RT-PCR for RVA identification was performed by using SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen™). Briefly, one-step RT-PCR was conducted in a final volume of 10 µl comprised of 1.5 µl of template RNA, 5 µl of 2xReaction Mix, 0.2 µl of 10 µM forward and reverse primers, 0.4 µl of SuperScript III RT (Invitrogen, CA) and distilled water to a final volume of 10 µl. The conditions of the RT-PCR assay included a cDNA synthesis step at 55°C for 30 min, an initial denaturation step at 94°C for 2 min, following by 40cycles of denaturation at 94°C for 30 s, annealing at 52°C for 45 s, and extension at 68°C for 1 min, as well as a final extension step at 68°C for 5 min. PCR product was run in gel electrophoresis and the expected product size of canine and feline rotavirus A viruses was 209 bp. Figure 6 showed example of RT-PCR result for the detection of RVA.

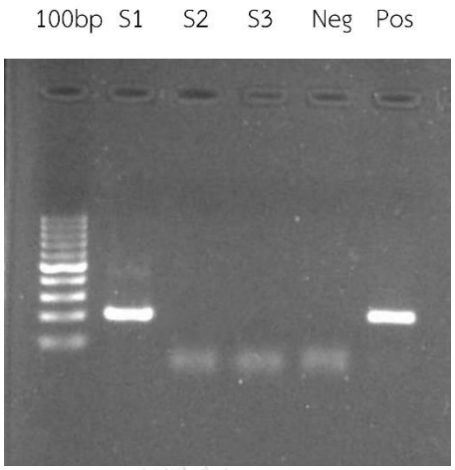


Figure 6: Detection of RVA by SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen™)

Lane 1; 100bp DNA ladder, Lane 2; Sample 1, Lane 3; Sample 2, Lane 4; Sample 3, Lane 5; Negative control, Lane 6; Positive control

3.3 Phase 3: Genetic characterization of RVA in dogs and cats by whole genome sequencing

3.3.1 Characterization of canine and feline rotavirus A viruses

3.3.1.1 Amplification of canine and feline rotavirus A

In this study, the canine and feline rotavirus A viruses were subjected to whole-genome sequencing. To perform whole genome sequencing, each gene of RVA (VP1, VP2, VP3, VP4, VP6, VP7, NSP1, NSP2, NSP3, NSP4, NSP5) was amplified by using primers previously described. The primer sequence and size of amplified products of each gene are present in Table 1. Briefly, one-step RT-PCR was conducted by using SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen™) in a final volume of 10 µl comprised of 1.5 µl of template RNA, 5 µl of 2xReaction Mix, 0.2 µl of 10 µM forward and reverse primers, 0.4 µl of SuperScript III RT (Invitrogen, CA) and distilled water to a final volume of 10 µl. The conditions of the RT-PCR assay included a cDNA synthesis step at 55°C for 30 min, an initial denaturation step at 94°C for 2 min, following by 40cycles of denaturation at 94°C for 30 s, annealing at 45-53°C for 45 s, and extension at 68°C for 1-4 min, as well as a final extension step at 68°C for 5 min. PCR reaction mixture and

PCR condition are shown in Table 2, Table 3, and Table 4. Figure 7 shows example of PCR product of each gene segment and corresponding PCR product size. PCR products of each gene were then pooled together and purified by NucleoSpin® Gel and PCR Clean-up (MACHEREY-NAGEL™, Germany). In detail, approximately 7 µl of PCR product from each primer set were pooled together in 1.5 ml Eppendorf, 200 µl of NTI buffer was put to mix with the pooled PCR product for the clean-up process, then all the mixture was transferred to the spin column and centrifuged at 11,000g for 30 sec. for DNA binding process, after discard the filtrated, 630 µl of NT3 buffer was added for the silica membrane washing process, the mixture was centrifuged at 11,000g for 30 sec., after 2 times washed and discard the filtrated in each time, the spin column was centrifuged in 11,000g for 1 min for silica membrane drying process. Last step was the eluted process by adding 15 µl of elution buffer into the spin column, incubated at room temperature for 1 min. and centrifuged at 11,000g for 1 min. The eluted process was done for 2 times in each sample, so the final volume of purified PCR product was approximately 30 µl for each sample. The purified PCR products were subjected for sequencing by Oxford Nanopore sequencing device and Minion flow cells with Rapid sequencing kit (ONT, UK).

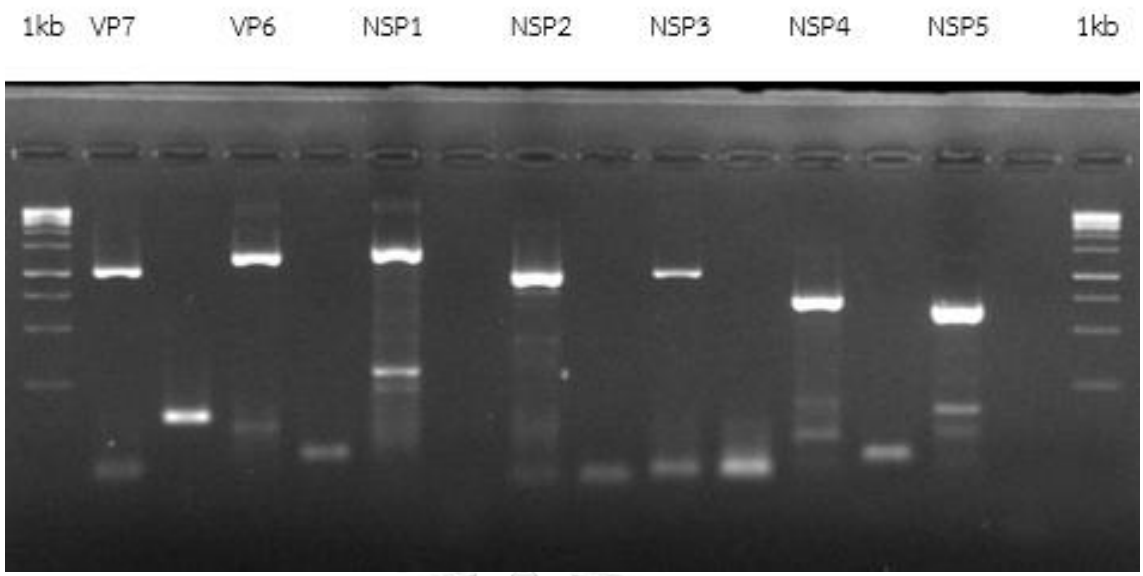


Figure 7: Example of PCR product for sequencing of each gene segment

Lane 1; 1kb DNA ladder, Lane 2; PCR product of VP7 gene, Lane 3; Negative control of VP7 gene, Lane 4; PCR product of VP6 gene, Lane 5; Negative control of VP6 gene, Lane 6; PCR product of NSP1 gene, Lane 7; Negative control of NSP1 gene, Lane 8; PCR product of NSP2 gene, Lane 9; Negative control of NSP2 gene, Lane 10; PCR product of NSP3 gene, Lane 11; Negative control of NSP3 gene, Lane 12; PCR product of NSP4 gene, Lane 13; Negative control of NSP4 gene, Lane 14; PCR product of NSP5 gene, Lane 15; Negative control of NSP5 gene, Lane 16; 1kb DNA ladder.

Table 1: List of sequencing primers used in this study

Primer name	Type	Sequence	Gene	Position(nt)	Product size(bp)	Species	Reference
RVA_VP1F_out	F	GGCTATTWAAGCTGTACAATGG	VP1	1-3300	3300	Dog, Cat	Charoenkul et al. 2020
RVA_VP1R_out	R	GGTCACATCYAAGCACTC					
RVA_VP2F_out	F	GGCTATTAAAGGYTCAATG	VP2	1-2700	2700	Dog, Cat	Charoenkul et al. 2020
RVA_VP2R_out	R	GGTCATATCTCCACAGTG					
RVA_VP3F_out	F	GGCTWTTAAAGCAGTATGAG	VP3	1-2581	2581	Dog, Cat	Charoenkul et al. 2020
RVA_VP3R_out	R	GGTCAMATCGTGACTAG					
RVA_VP4F_out	F	GGCTATAAAATGGCTTCGCTCATT	VP4	1-2300	2300	Dog, Cat	Charoenkul et al. 2020
RVA_VP4R_out	R	GGTCACATCCTCTAGAAATTGC					
RVA_VP6F_out	F	GGCTTTTAAACGAAGTCTTC	VP6	1-1350	1350	Dog, Cat	Charoenkul et al. 2020
RVA_VP6R_out	R	GGTCACATCCTCTCACT					
RVA_VP7F_out	F	GGCTTTAAAAGCGAGAATTTCCGT	VP7	1-1036	1036	Dog, Cat	Charoenkul et al. 2020
RVA_VP7R_out	R	GGTCACATCATACAATTCTAA					
RVA_NSP1F_out	F	GGCTTTTTTTTGAAAAGTCTTG	NSP1	1-1500	1500	Dog, Cat	Charoenkul et al. 2020
RVA_NSP1R_out	R	GGTTCACAGTTTTTGCTGGCTAGG					
RVA_NSP2F_out	F	GGCTTTTAAAGCGTCTCAG	NSP2	1-1021	1021	Dog, Cat	Charoenkul et al. 2020
RVA_NSP2R_out	R	GGTCACATAAGCGCTTTC					
RVA_NSP3F_out	F	GGCTTTTAATGCTTTTCAGTG	NSP3	1-1100	1100	Dog, Cat	Charoenkul et al. 2020
RVA_NSP3R_out	R	GGTCACATAACGCCCCTATAGC					

Primer name	Type	Sequence	Gene	Position(nt)	Product size(bp)	Species	Reference
RVA_NSP4F_out	F	GGCTTTTAAAAGTTCTGTTCCG	NSP4	1-750	750	Dog, Cat	Charoenkul et al. 2020
RVA_NSP4R_out	R	GGTCACAYAAAGACCGTTCCTTCC					
RVA_NSP5F_out	F	GGCTTTTAAAGCGCTACAG	NSP5	1-780	780	Dog, Cat	Charoenkul et al. 2020
RVA_NSP5R_out	R	GGTCACAAAACGGGAGT					
RVA_VP4_con3	F	TGGCTTCGCTCATTTATAGACA	VP4	31-871	840	Dog, Cat	Gentsch et al. 2014
RVA_VP4_con2	R	ATTTCGGACCATTTATAACC					

Table 2: Annealing temperature and extension time for each pair of primer used in PCR reaction

Primer name	Type	Sequence	Gene	Product size (bp)	Annealing temp. (°C)	Extension time (min.)	Reference
RVA_VP1F_out	F	GGCTATTWAAGCTGTACAATGG	VP1	3300	48°C	4 min	Charoenkul et al. 2020
RVA_VP1R_out	R	GGTCACATCYAAGCACTC					
RVA_VP2F_out	F	GGCTATTAAAGGYTCAATG	VP2	2700	48°C	3 min	Charoenkul et al. 2020
RVA_VP2R_out	R	GGTCATATCTCCACAGTG					
RVA_VP3F_out	F	GGCTWTTAAAGCAGTATGAG	VP3	2581	48°C	3 min	Charoenkul et al. 2020
RVA_VP3R_out	R	GGTCAMATCGTGACTAG					
RVA_VP4F_out	F	GGCTATAAAATGGCTTCGCTCATT	VP4	2300	48°C	3 min	Charoenkul et al. 2020
RVA_VP4R_out	R	GGTCACATCCTCTAGAAATTGC					
RVA_VP6F_out	F	GGCTTTTAAACGAAGTCTTC	VP6	1350	48°C	2 min	Charoenkul et al. 2020
RVA_VP6R_out	R	GGTCACATCCTCTCACT					
RVA_VP7F_out	F	GGCTTTAAAAGCGAGAATTTCCGT	VP7	1036	48°C	1.30 min	Charoenkul et al. 2020
RVA_VP7R_out	R	GGTCACATCATACAATTCTAA					
RVA_NSP1F_out	F	GGCTTTTTTTTGAAAAGTCTTG	NSP1	1500	50°C	2 min	Charoenkul et al. 2020
RVA_NSP1R_out	R	GGTTCACAGTTTTTGCTGGCTAGG					
RVA_NSP2F_out	F	GGCTTTTAAAGCGTCTCAG	NSP2	1021	48°C	1.30 min	Charoenkul et al. 2020
RVA_NSP2R_out	R	GGTCACATAAGCGCTTTC					
RVA_NSP3F_out	F	GGCTTTTAATGCTTTTCAGTG	NSP3	1100	50°C	1.30 min	Charoenkul et al. 2020
RVA_NSP3R_out	R	GGTCACATAACGCCCCTATAGC					
RVA_NSP4F_out	F	GGCTTTTAAAAGTTCTGTTCCG	NSP4	750	53°C	1 min	Charoenkul et al. 2020
RVA_NSP4R_out	R	GGTCACAYAAAGACCGTTCCTTCC					

Primer name	Type	Sequence	Gene	Product size (bp)	Annealing temp. (°C)	Extension time (min.)	Reference
RVA_NSP5F_out	F	GGCTTTTAAAGCGCTACAG	NSP5	780	48°C	1 min	Charoenkul et al. 2020
RVA_NSP5R_out	R	GGTCACAAAACGGGAGT					
RVA_VP4_con3	F	TGGCTTCGCTCATTTATAGACA	VP4	840	45°C	1 min	Gentsch et al. 2014
RVA_VP4_con2	R	ATTTCGGACCATTTATAACC					

Table 3: PCR reaction mixture for RVA detection and sequencing used in this study

Mixture	Amount
RNA	1.5 µl
Forward primer	0.2 µl
Reverse primer	0.2 µl
2X reaction mix	5 µl
SuperScript III RT (Invitrogen, CA)	0.4 µl
Distilled water	2.7 µl
Total	10 µl

Table 4: PCR condition for RVA detection and sequencing used in this study

Step	Temp (°C)	Time	Cycle
cDNA synthesis	55	30 min	1
Initial denaturation	94	2 min	1
Denaturation	94	30 sec	40
Annealing	45-53	45 sec	
Extension	68	1-4 min	
Final extension	68	5 min	1

3.3.1.2 Whole genome sequencing of RVA by nanopore sequencing technique

The whole genome sequencing of RVA was done by using MinION oxford nanopore sequencing device and MinION flow cells. The rapid sequencing kit (Cat#SQK-RAD004) was used to prepare the DNA library and the flow cells priming mix following manufacturer's instruction. In detail, first, the number of pores in flow cell were checked via the MinKNOW software to ensure the ability of sequencing, the number of pores should not be lower than 200 to ensure the quality of sequences. To prepare the DNA library for loading into flow cell, 7.5µl of purified PCR product were mixed with 2.5µl of fragmentation mix (FRA) and incubated in thermocycler at

30°C for 1 min and then at 80°C for 1 min and cool on ice, 1µl of rapid adapter (RAP) was added and incubated at room temperature for 5 min. 34µl of sequencing buffer (SQB), 25.5µl of loading beads (LB), 4.5µl of nuclease-free water were added to complete the loading DNA library. The flow cell priming mix was prepared by the mix of 30µl flush tether (FLT) with the flush buffer (FB). To start sequencing, first, 800µl of prepared flow cell priming mix was loaded to the flow cell and incubated for 5 min, then 200µl more of flow cell priming mix was loaded to the flow cell and then 75µl of prepared DNA library were followed, and the sequencing process was started via MinKNOW software. The MinION oxford nanopore sequencing device and MinION flow cells are shown in Figure 8. The MinKNOW software and Nanopore sequencing procedure is shown in Figure 9.

Figure 8: MinION oxford nanopore sequencing device and MinION flow cell

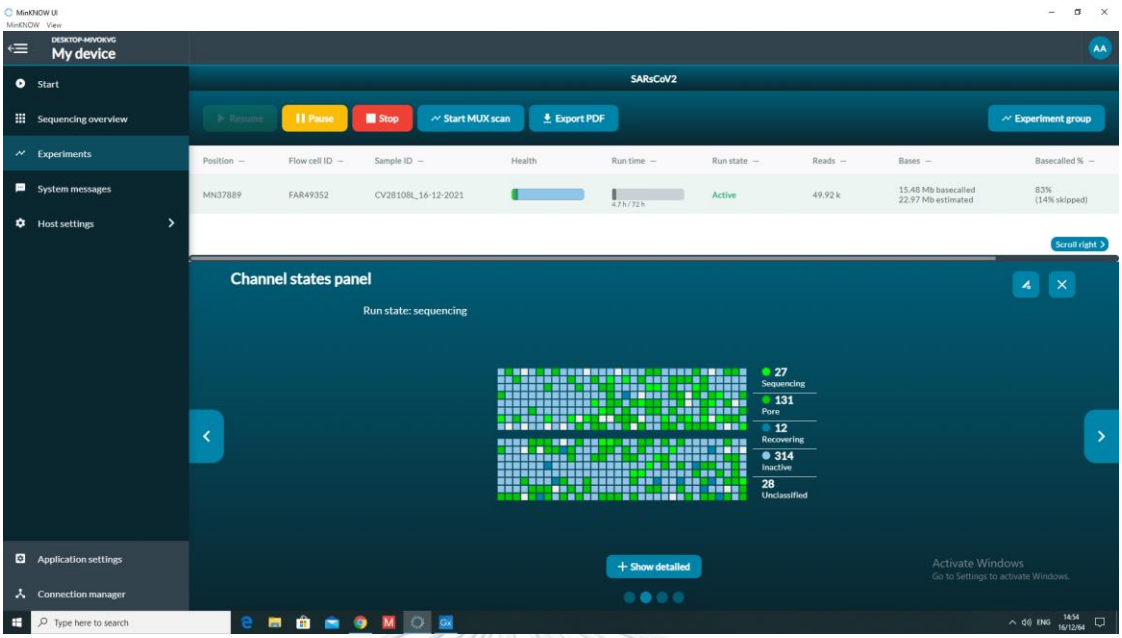


Figure 9: The MinKNOW software and Nanopore sequencing procedure

3.3.1.3 Validation and assembly of whole genome sequences of rotavirus A viruses

The Minknow software was used for sequence reading and basecalling process to converse the electrical signal (fast5 file) to nucleotide (fastq file), the minimum Qscore 7 was used to remove the poor-quality sequence from basecalling process. The nucleotide sequences were then assembled using de-novo assembly by genome detective web software (https://www.genomedetective.com/app/typingtool/virus) (Figure 10). The fastq file from MinKNOW software were uploaded into the web for process of de-novo assembly and mapped with reference RVAs in the web database. The nucleotide sequence of each gene segment of RVAs were retrieved in fasta file format. The fasta format nucleotide sequences of each gene segment were then BLAST with NCBI database to find the closest identity sequence to be the reference of each gene segment. Finally, CLC genomic Workbench software 20.0 (Qiagen, Hilden, Germany) was used to map the original fastq file from MinKNOW software with the reference sequences by function map read to reference of the software (Example of report of map to reference function by CLC genomic Workbench software in Figure 11), at last,

the consensus sequence of each gene segment of the viruses were exported in the fasta file format for further analysis.

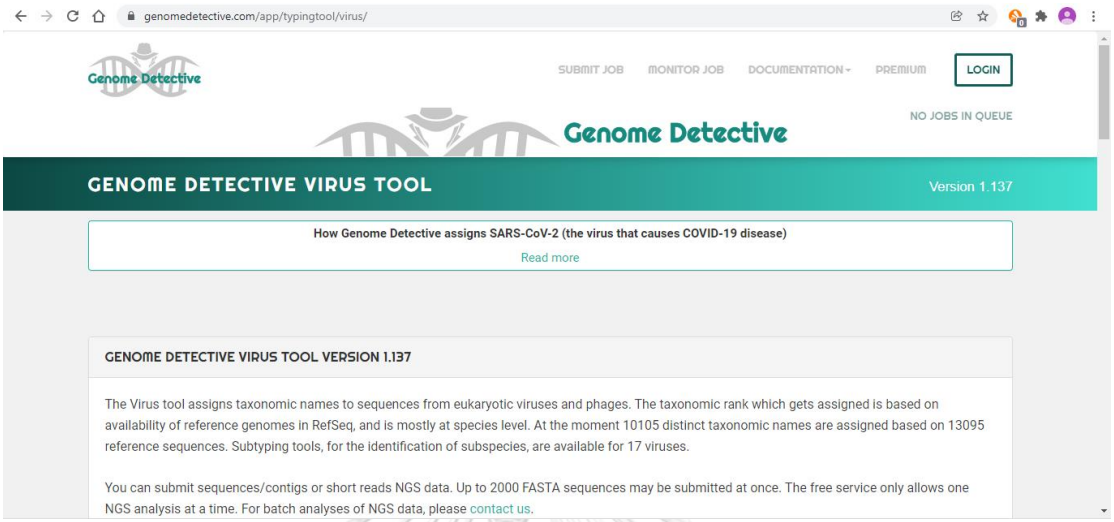


Figure 10: Genome detective web software used for de-novo assembly

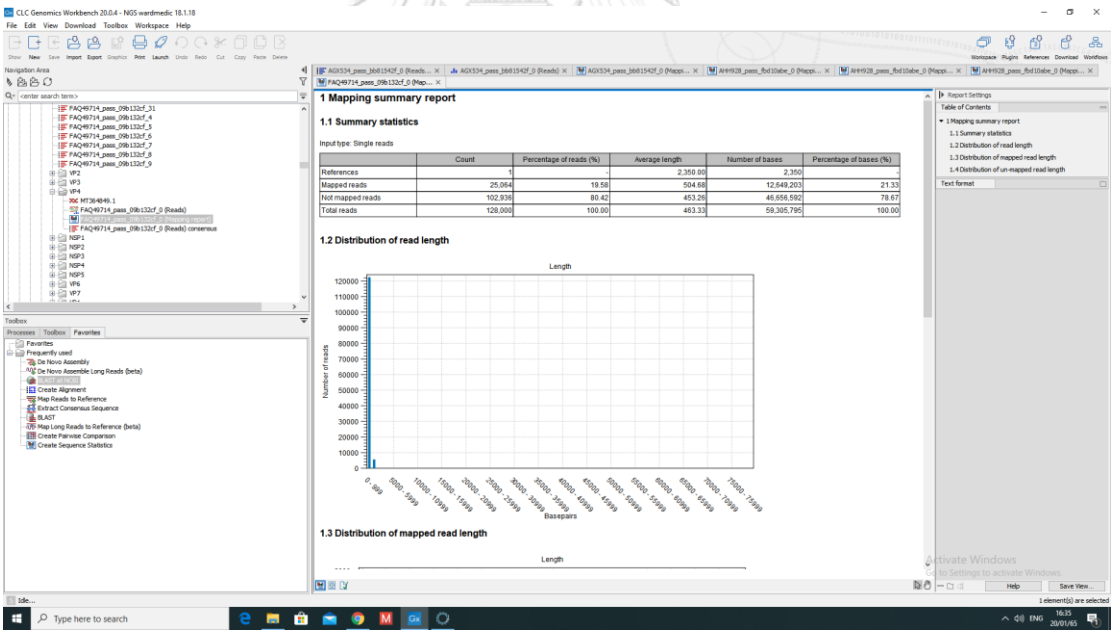


Figure 11: Example of map to reference report by CLC genomic software

3.3.2 Genetic diversity of RVA

3.3.2.1 Genotyping of rotavirus A viruses

To identify genotype of RVA, nucleotide sequence of each segment were analyzed by the Virus pathogen resource (ViPR) web software

(https://www.viprbrc.org/brc/rvaGenotyper.spg?method=ShowCleanInputPage&decorator=reo) (Figure 12). The algorithm is based on the RotaC algorithm developed by Maes et al., 2009. Briefly, fasta file format of each gene segment were uploaded into the ViPR web program to identify and designate the genotype of RVA. The example of genotyping result is shown in Figure 13.

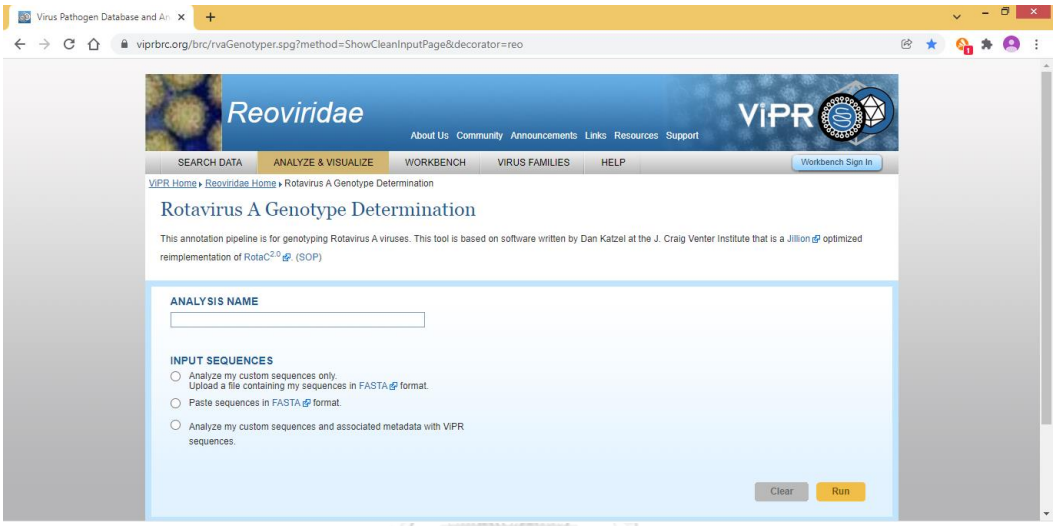
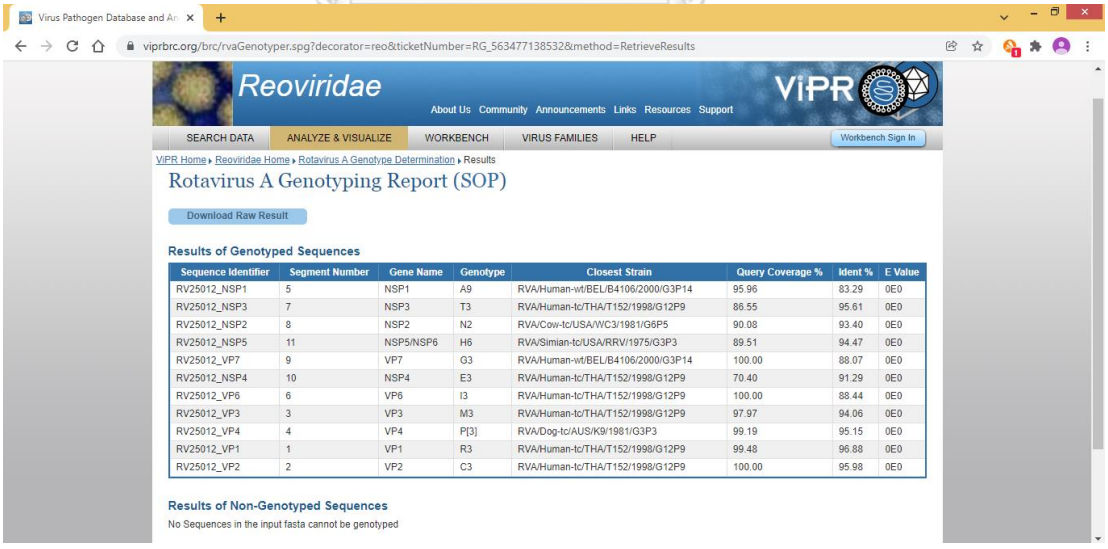


Figure 12: Virus pathogen resource (ViPR) web software use for genotyping of RVA



Sequence Identifier	Segment Number	Gene Name	Genotype	Closest Strain	Query Coverage %	Ident %	E Value
RV25012_NSP1	5	NSP1	A9	RVA/Human-wt/BEL/B4106/2000/G3P14	95.96	83.29	0E0
RV25012_NSP3	7	NSP3	T3	RVA/Human-tc/THA/T152/1998/G12P9	86.55	95.61	0E0
RV25012_NSP2	8	NSP2	N2	RVA/Cow-tc/USA/WC3/1981/G6P5	90.08	93.40	0E0
RV25012_NSP5	11	NSP5/NSP6	H6	RVA/Simian-tc/USA/RRV/1975/G3P3	89.51	94.47	0E0
RV25012_VP7	9	VP7	G3	RVA/Human-wt/BEL/B4106/2000/G3P14	100.00	88.07	0E0
RV25012_NSP4	10	NSP4	E3	RVA/Human-tc/THA/T152/1998/G12P9	70.40	91.29	0E0
RV25012_VP6	6	VP6	I3	RVA/Human-tc/THA/T152/1998/G12P9	100.00	88.44	0E0
RV25012_VP3	3	VP3	M3	RVA/Human-tc/THA/T152/1998/G12P9	97.97	94.06	0E0
RV25012_VP4	4	VP4	P[3]	RVA/Dog-tc/AUS/K9/1981/G3P3	99.19	95.15	0E0
RV25012_VP1	1	VP1	R3	RVA/Human-tc/THA/T152/1998/G12P9	99.48	96.88	0E0
RV25012_VP2	2	VP2	C3	RVA/Human-tc/THA/T152/1998/G12P9	100.00	95.98	0E0

Figure 13: Example of genotyping result from Virus pathogen resource (ViPR) web software

3.3.2.2 Phylogenetic analysis of rotavirus A viruses

Phylogenetic tree of each segment (11 segments) was generated by MEGA v10. 0 software with neighbor-joining algorithm and bootstrap analysis of 1,000 replications (Tamura et al., 2013). The nucleotide sequences of references RVAs from dogs, cats, humans, pigs, horses, and bats were selected to include in phylogenetic analysis. RVA sequences from this study and reference sequences were aligned by ClustalW function and only the coding region of each segment of RVA were used to generate the phylogenetic tree of each segment of the virus by using MEGA software (Figure 14). The example of phylogenetic analysis from MEGAX is shown in Figure 15.

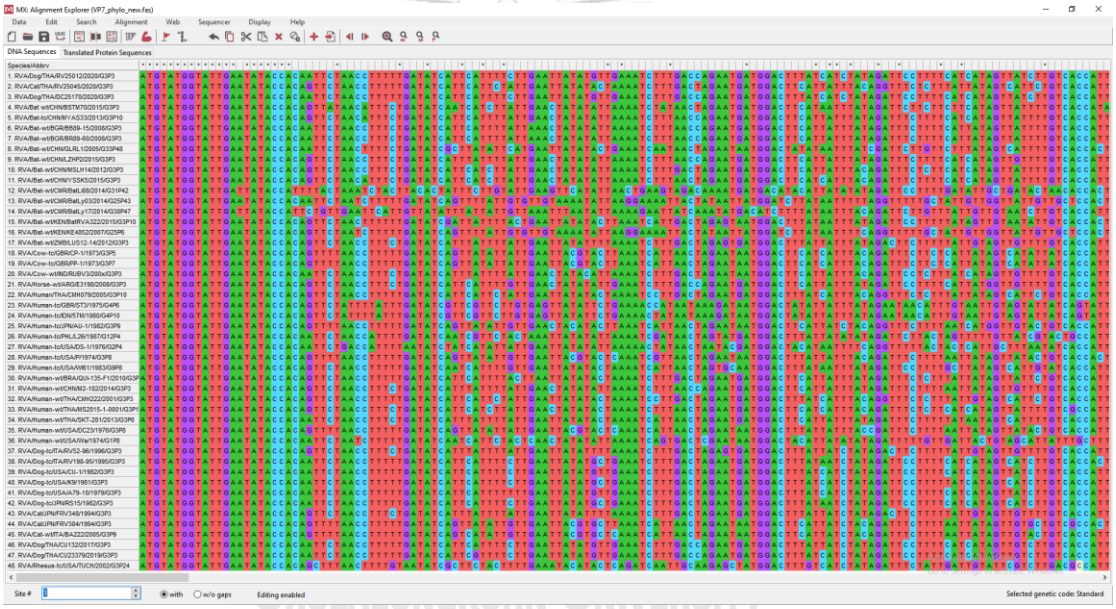


Figure 14: Sequence alignment in MEGAX for phylogenetic analysis

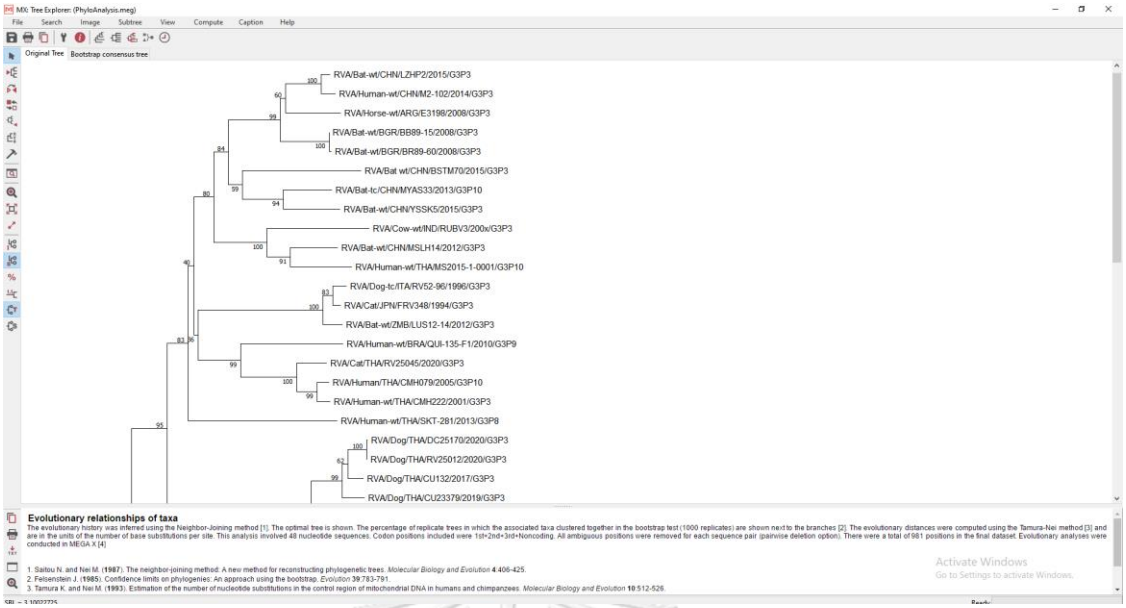
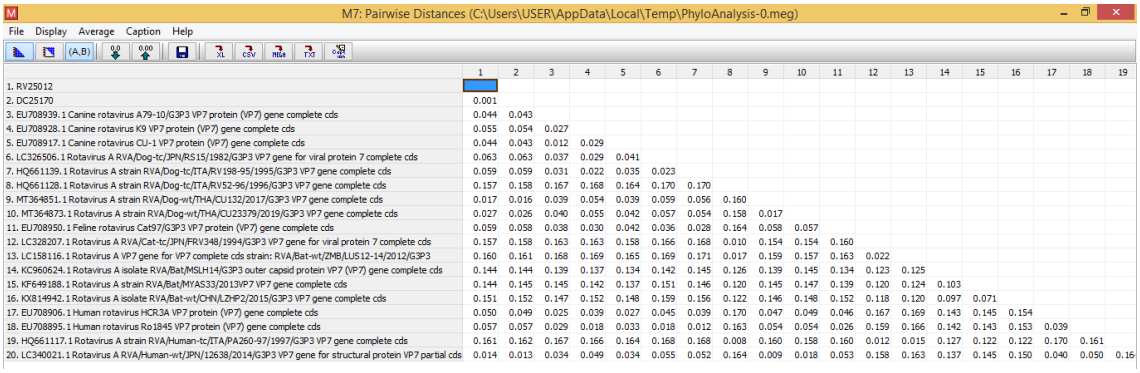


Figure 15: Example of phylogenetic analysis from MEGAX

3.3.2.3 Nucleotide and amino acid identities

The percentages of nucleotide and amino acid identities of each gene segment between RVAs in this study and closely related strains from phylogenetic analysis, as well as some other reference strains within the same genotype were calculated using pairwise distance function in MEGA v10.0 software. The nucleotide sequences of RVAs in this study along with closely related RVA strains and reference strains were aligned by ClustalW function and only the coding region of each segment of RVA were used to calculate the percentage of similarity of each segment of the virus by using MEGA software. The example of pairwise distance calculation from MEGAX is shown in Figure 16.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. RV25012																			
2. DC25170	0.001																		
3. EU708939.1 Canine rotavirus A79-10/G3P3 VP7 protein (VP7) gene complete cds	0.044	0.043																	
4. EU708928.1 Canine rotavirus K9 VP7 protein (VP7) gene complete cds	0.055	0.054	0.027																
5. EU708917.1 Canine rotavirus CU-1 VP7 protein (VP7) gene complete cds	0.044	0.043	0.012	0.029															
6. LC326506.1 Rotavirus A RVA/Dog-tc/JPN/RS15/1982/G3P3 VP7 gene for viral protein 7 complete cds	0.063	0.063	0.037	0.029	0.041														
7. HQ661139.1 Rotavirus A strain RVA/Dog-tc/ITA/RV198-95/1995/G3P3 VP7 gene complete cds	0.059	0.059	0.031	0.022	0.035	0.023													
8. HQ661128.1 Rotavirus A strain RVA/Dog-tc/ITA/RV52-96/1996/G3P3 VP7 gene complete cds	0.157	0.158	0.167	0.168	0.164	0.170	0.170												
9. MT364851.1 Rotavirus A strain RVA/Dog-wt/THA/CU132/2017/G3P3 VP7 gene complete cds	0.017	0.016	0.039	0.054	0.039	0.059	0.056	0.160											
10. MT364873.1 Rotavirus A strain RVA/Dog-wt/THA/CU23379/2019/G3P3 VP7 gene complete cds	0.027	0.026	0.040	0.055	0.042	0.057	0.054	0.158	0.017										
11. EU708950.1 Feline rotavirus Cat97/G3P3 VP7 protein (VP7) gene complete cds	0.059	0.058	0.038	0.030	0.042	0.036	0.028	0.164	0.058	0.057									
12. LC328207.1 Rotavirus A RVA/Cat-tc/JPN/FRV348/1994/G3P3 VP7 gene for viral protein 7 complete cds	0.157	0.158	0.163	0.163	0.158	0.166	0.168	0.010	0.154	0.154	0.160								
13. LC158116.1 Rotavirus A VP7 gene for VP7 complete cds strain: RVA/Bat-wt/ZMB/LUS12-14/2012/G3P3	0.160	0.161	0.168	0.169	0.165	0.169	0.171	0.017	0.159	0.157	0.163	0.022							
14. KC960624.1 Rotavirus A isolate RVA/Bat/MSLH14/G3P3 outer capsid protein VP7 (VP7) gene complete cds	0.144	0.144	0.139	0.137	0.134	0.142	0.145	0.126	0.139	0.145	0.134	0.123	0.125						
15. KF649188.1 Rotavirus A strain RVA/Bat/MYAS33/2013VP7 VP7 gene complete cds	0.144	0.145	0.145	0.142	0.137	0.151	0.146	0.120	0.145	0.147	0.139	0.120	0.124	0.103					
16. KX814942.1 Rotavirus A isolate RVA/Bat-wt/CHN/LZHP2/2015/G3P3 VP7 gene complete cds	0.151	0.152	0.147	0.152	0.148	0.159	0.156	0.122	0.146	0.148	0.152	0.118	0.120	0.097	0.071				
17. EU708906.1 Human rotavirus HCR3A VP7 protein (VP7) gene complete cds	0.050	0.049	0.025	0.039	0.027	0.045	0.039	0.170	0.047	0.049	0.046	0.167	0.169	0.143	0.145	0.154			
18. EU708895.1 Human rotavirus Ro1845 VP7 protein (VP7) gene complete cds	0.057	0.057	0.029	0.018	0.033	0.018	0.012	0.163	0.054	0.054	0.026	0.159	0.166	0.142	0.143	0.153	0.039		
19. HQ661117.1 Rotavirus A strain RVA/Human-tc/ITA/PA260-97/1997/G3P3 VP7 gene complete cds	0.161	0.162	0.167	0.166	0.164	0.168	0.168	0.008	0.160	0.158	0.160	0.012	0.015	0.127	0.122	0.122	0.170	0.161	
20. LC340021.1 Rotavirus A RVA/Human-wt/JPN/12638/2014/G3P3 VP7 gene for structural protein VP7 partial cds	0.014	0.013	0.034	0.049	0.034	0.055	0.052	0.164	0.009	0.018	0.053	0.158	0.163	0.137	0.145	0.150	0.040	0.050	0.16

Figure 16: Example of pairwise distance calculation from MEGAX

3.3.2.4 Genetic constellation of RVA

From genotyping of each gene segment of RVA, the combination of 11 genotypes from each gene segment were analyzed and assigned as genetic constellation. The genetic constellation of RVAs in this study were compared with closely related strains from phylogenetic analysis. The reference strains of RVAs from different species which include dog, cat, pig, horse, bat, and human were selected to compare with viruses in this study. The reference strains which had the same genotype in almost segments of the virus or had the same or almost the same genetic constellation with viruses in this study were selected to include in this comparison. The example of genetic constellation showed in Figure 17.

	Strain names[a]	Genotype constellation	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
A	RVA/Human-tc/USA/Wa/1974/G1P1A[8]	Human Wa-like	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-tc/USA/P/1974/G3P1A[8]		G3	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/USA/DC5115-Bethesda/1977/G4P[8]		G4	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/BEL/B3458/2003/G9P[8]		G9	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/BEL/B4633/2003/G12P[8]		G12	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-tc/BRA/IAL28/1992/G5P[8]		G5	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/CIV/6736/2004/G8P[8]		*G8*	*P[8]*	*I1*	*R1*	*C1*	*M1*	*A1*	*N1*	*T1*	*E1*	*H1*
	RVA/Human-wt/NGA/6717ARN/2002/G10P[8]		*G10*	*P[8]*	*I1*	*R1*	*C1*	*M1*	*A1*	*N1*	*T1*	*E1*	H1
	RVA/Human-wt/BGD/Matlab36/2002/G11P[8]		G11	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
B	RVA/Human-tc/USA/DS-1/1976/G2P1B[4]	Human DS-1-like	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
	RVA/Human-wt/DEU/GER1H/2009/G8P[4]		G8	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
	RVA/Human-wt/BEL/B1711/2002/G6P[6]		G6	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2
	RVA/Human-wt/COD/DRC88/2003/G8P[8]		G8	P[8]	I2	R2	C2	M2	A2	N2	T2	E2	H2
	RVA/Human-wt/COD/DRC86/2003/G8P[6]		G8	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2
	RVA/Human-tc/IND/69M/1980/G8P4[10]		G8	P[10]	I2	R2	C2	M2	A2	N2	T2	E2	H2
	RVA/Human-wt/ZAF/GR10924/1999/G9P[6]		G9	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2
C	RVA/Human-tc/KEN/AK26/1982/G2P[4]	Wa-like x DS-1-like Reassortant	G2	P[4]	I2	R2	C2	M2	A2	N1	T2	E2	H2
	RVA/Human-wt/CAM/6809/2000/G8P[6]		*G8*	*P[6]*	*I2*	*R1*	*C1*	*M1*	*A1*	*N1*	*T1*	*E1*	*H1*
	RVA/Human-wt/CIV/6755ARN/2002/G10P[8]		*G10*	*P[8]*	*I2*	*R1*	*C1*	*M1*	*A1*	*N1*	*T1*	*E1*	H1
	RVA/Human-tc/PHL/L26/1987/G12P[4]		G12	P[4]	I2	R2	C2	M1/M2	A2	N1	T2	E2	H1
	RVA/Human-wt/ZAF/3133WC/2009/G12P[4]		G12	P[4]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/BGD/Matlab13/2003/G12P[6]		G12	P[6]	I1	R1	C1	M1	A1	N1	T2	E1	H1
	RVA/Human-wt/BGD/N26/2002/G12P[6]		G12	P[6]	I2	R2	C2	M2	A2	N1	T2	E6	H2
	RVA/Human-wt/BGD/RV161/2000/G12P[6]		G12	P[6]	I2	R2	C2	M2	A2	N2	T2	E1	H2
D	RVA/Human-tc/JPN/AU-1/1982/G3P3[9]	Human AU-1-like	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
	RVA/Human-tc/THA/T152/1998/G12P[9]		G12	P[9]	I3	R3	C3	M3	A12	N3	T3	E3	H6
E	RVA/Human-wt/IND/N155/2003/G10P[11]	Wa-like Human x Bovine Reassortant	G10	P[11]	I2	R2	C2	M2	A1	N1	T1	E2	H3
	RVA/Human-tc/IND/1321/XXXX/G10P[11]		G10	P[11]	I2	Rx	Cx	Mx	A1	N2	T1	E2	Hx
F	RVA/Human-tc/ITA/PA169/1988/G6P[14]	Human Bovine-like	G6	P[14]	I2	R2	C2	M2	A3	N2	T6	E2	H3
	RVA/Human-wt/HUN/Hun5/1997/G6P[14]		G6	P[14]	I2	R2	C2	M2	A11	N2	T6	E2	H3
	RVA/Human-wt/HUN/BP1062/2004/G8P[14]		G8	P[14]	I2	R2	C2	M2	A11	N2	T6	E2	H3
	RVA/Human-tc/GBR/A64/1987/G10P11[14]		G10	P[14]	I2	R2	C2	M1	A3	N2	T6	E2	H3
G	RVA/Cow-tc/FRA/RF/1982/G6P[1]	Artiodactyl Bovine-like	G6	P[1]	I2	R2	C2	M2	A3	N2	T6	E2	H3
	RVA/Sheep-tc/ESP/OVR762/2002/G8P[14]		G8	P[14]	I2	R2	C2	M2	A11	N2	T6	E2	H3
	RVA/Guanaco-wt/ARG/Chubut/1999/G8P[14]		G8	P[14]	I2	R5	C2	M2	A11	N2	T6	E12	H3
	RVA/Antelope-wt/ZAF/RC-18/2008/G6P[14]		G6	P[14]	I2	R2	C2	M2	A11	N2	T6	E2	H3
	RVA/Goat-tc/BGD/GO34/1999/G6P[1]		G6	P[1]	I2	R2	C2	M2	A11	N2	T6	E2	H3
H	RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P7[5]	Vaccine	G1	P[5]	I2	R2	C2	M1	A3	N2	T6	E2	H3
	RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P7[5]		G2	P[5]	I2	R2	C2	M1	A3	N2	T6	E2	H3
	RVA/Vaccine/USA/RotaTeq-WI78-8/1992/G3P7[5]		G3	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
	RVA/Vaccine/USA/RotaTeq-BrB-9/1996/G4P7[5]		G4	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
	RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A[8]		G6	P[8]	I2	R2	C2	M2	A3	N2	T6	E2	H3
	RVA/Vaccine/USA/Rotarix-RIX4414/1988/G1P1A[8][c]		G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1

Figure 17: Example of genetic constellation of RVAs
(Matthijnssens and Van Ranst, 2012)

3.3.2.5 Reassortment analysis of RVA

The vistaplot and bootscan analysis were done to detect the reassortment events of RVAs in this study. The vistaplot analysis was generated by wgVISTA web software (https://genome.lbl.gov/cgi-bin/WGVistaInput) to compare the RVAs in this study with closely related strains (Frazer et al., 2004, Mayor et al., 2000). In brief, the DNA sequences of coding region from all 11 segments of each RVA in this study and closely related strains were prepared as a concatenated sequence, the concatenated sequence of RVA in this study was then uploaded in the wgVISTA web software together with closely related strains to compared for similarity between the concatenated sequence. The example of vistaplot analysis result showed in Figure 18. For bootscan analysis, the SimPlot v.3.5.1 program were used in this study. In brief, the concatenated DNA sequence of RVAs in this study and closely related strains were input in the SimPlot v.3.5.1 program and analyzed by bootscan function with neighbor-joining model, 1000 bootstrap replicates, Kimura (2-parameter) distance model, 1000bp window size, and 70bp step size. The example of bootscan analysis result showed in Figure 19.

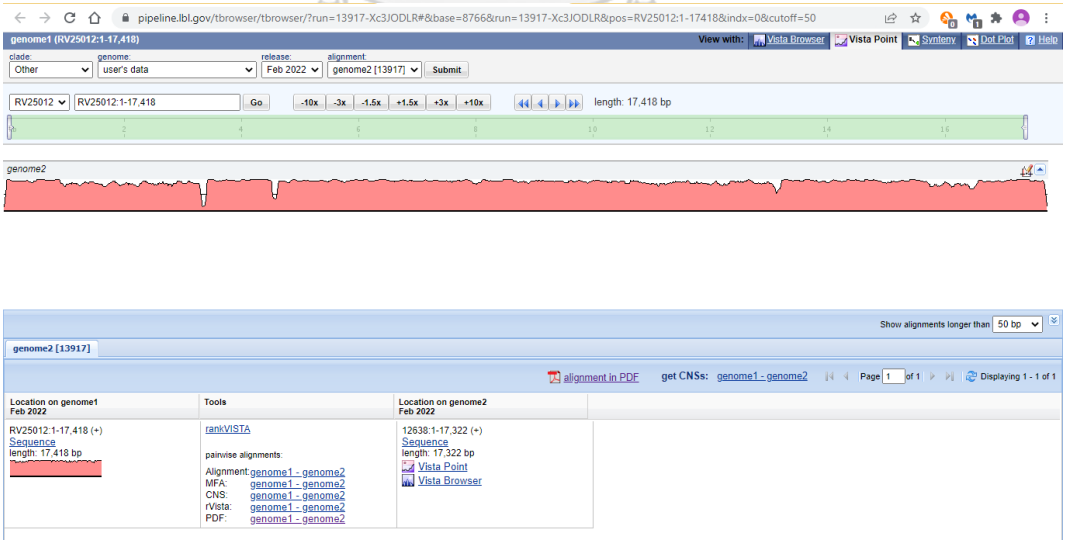


Figure 18: Example of vistaplot analysis from wgVISTA web software

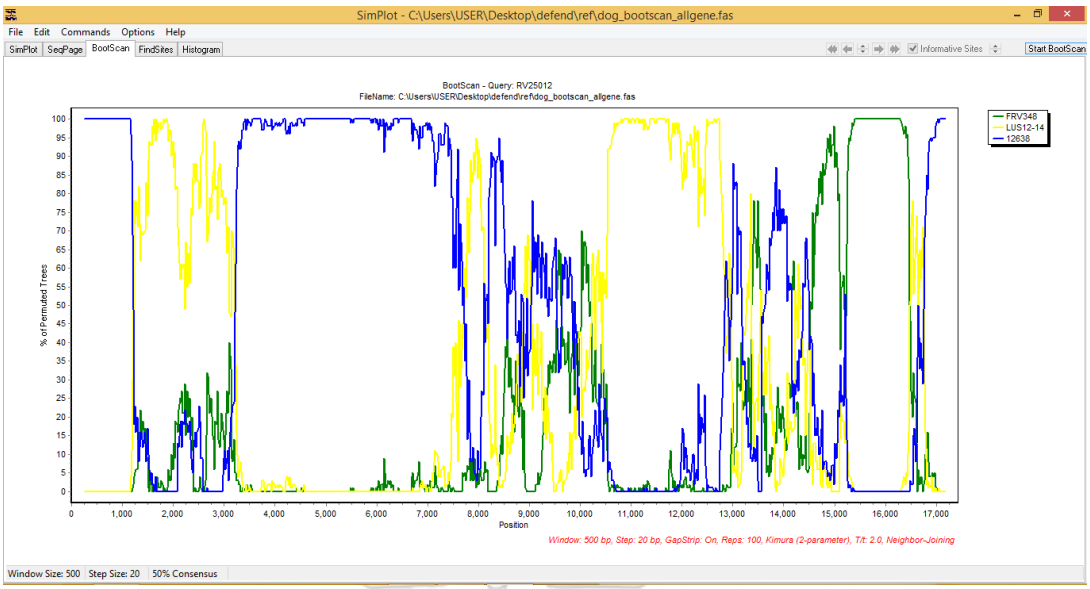


Figure 19: Example of bootscan analysis from SimPlot

Chapter 4
Result

The results of this thesis are provided corresponding to three study phases

4.1 Sample collection from dogs and cats in animal hospitals in Bangkok and the vicinities

In this thesis, we collected rectal swab samples from dogs and cats from 8 animal hospitals in Bangkok (n=5) and Nonthaburi (n=3). In total 572 rectal swab samples were included in this study. Five animal hospitals in Bangkok located in Huai-Khwang district, Bang-Kho-Laem district, Taling-Chan district, Lak-Si district, and Yan-Nawa district. Three animal hospitals in Nonthaburi were located in Mueang Nonthaburi district (Table 5).

By province, out of 572 samples, 193 and 192 rectal swab samples were collected from dogs and cats from animal hospitals in Bangkok, respectively. While 97 and 90 rectal swab samples were collected from dogs and cats from animal hospitals in Nonthaburi, respectively (Table 5 and Figure 20).

By year, rectal swab samples of dogs (n=290), 108 samples were collected in 2020, and 182 samples were collected in 2021. For rectal swab samples of cats, 105 samples were collected in 2020, and 177 samples were collected in 2021. The description of rectal swab samples by month/year is shown in Table 6 and Figure 21.

By age of animals, we have categorized dogs and cats into 5 age groups according to life stage of the animals including 1) puppy and kitten stage (<6 months), 2) junior life stage (6-<12 months), 3) adult life stage (1-<6 years), 4) mature life stage (6-<10 years), and 5) senior life stage (≥10 years) (Vogt et al., 2010; Bartges et al., 2012). For puppy and kitten stage (first born to less than 6 months), 64 dog and 59 cat samples were included in this study. For junior life stage (6 months to less than 12 months), there were 22 dogs and 45 cats. For adult life stage (1 year to less than 6 years), there were 63 dogs and 83 cats. For mature life stage (6 years to less than 10 years), there were 35 dogs and 15 cats. For senior life stage (10 years or

more), there were 49 dogs and 15 cats. It is noted that, there were some dogs (n=57) and cats (n=65) with uncertain specific age, mostly the adopted dogs or cats (Table 7 and Figure 22).

By sex of animals, the rectal swab samples were collected from 160 male and 101 female dogs, as well as 140 male and 130 female cats. There were 29 dogs, and 12 cats did not have the information of sex (Table 8 and Figure 23).

Table 5: Description of rectal swab samples collected by district and province

Species	Province						Total
	Bangkok					Nonthaburi	
	Huai-Khwang	Bang-Kho-Laem	Taling-Chan	Lak-Si	Yan-Nawa		
Canine	105	27	19	39	3	97	290
Feline	141	9	8	16	18	90	282

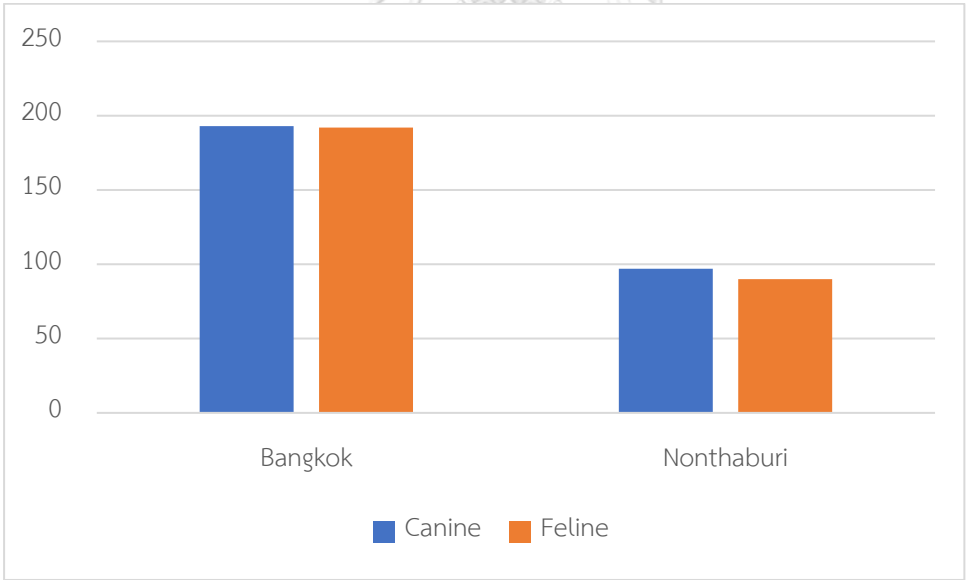


Figure 20: Bar charts of rectal swab samples collected by province

Table 6: Description of rectal swab samples collected by month

Species	2020									2021						Total
	Jan	Feb	Mar	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	
Canine	16	1	4	12	14	6	20	7	28	12	0	45	39	34	52	290
Feline	0	8	8	5	14	17	22	12	19	18	4	45	36	33	41	282

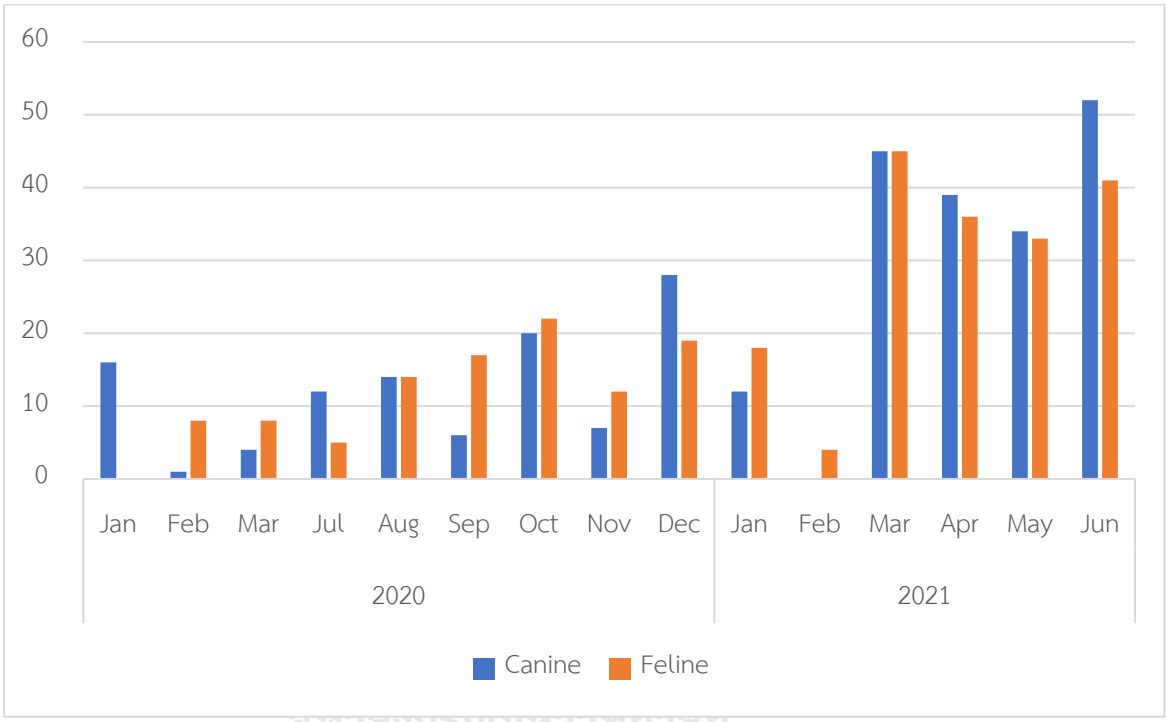


Figure 21: Bar charts of rectal swab samples collected by month

Table 7: Description of rectal swab samples collected by age

Species	Age						Total
	Puppy/Kitten 0 - < 6 mths	Junior 6 - < 12 mths	Adult 1 - < 6 yrs	Mature 6 - < 10 yrs	Senior ≥ 10 yrs	NA	
Canine	64	22	63	35	49	57	290
Feline	59	45	83	15	15	65	282

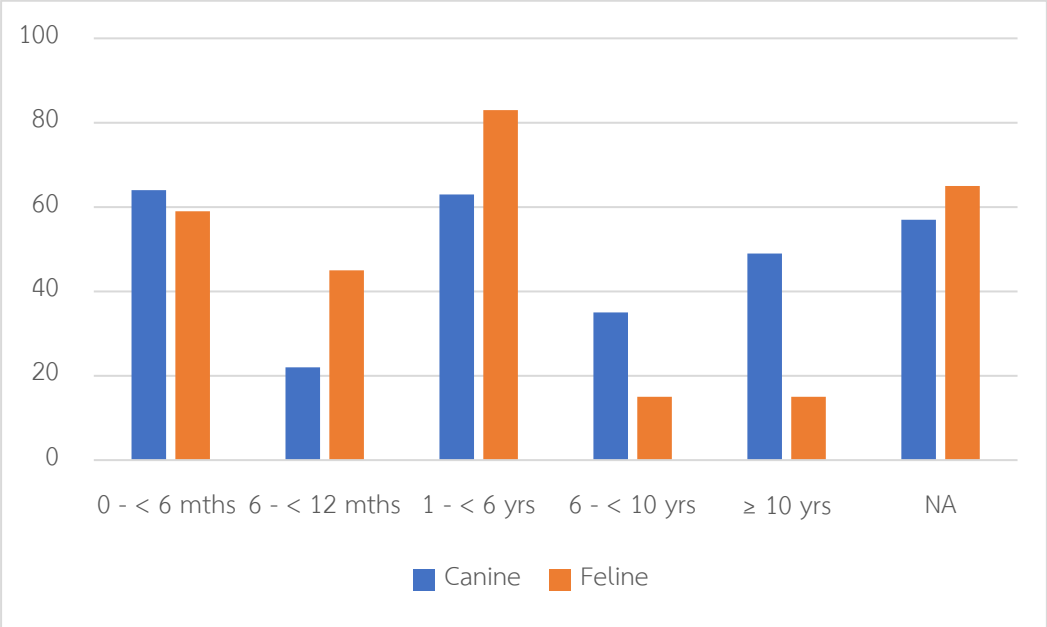


Figure 22: Bar charts of rectal swab samples collected by age

Table 8: Description of rectal swab samples collected by sex

Species	Sex			Total
	Male	Female	NA	
Canine	160	101	29	290
Feline	140	130	12	282

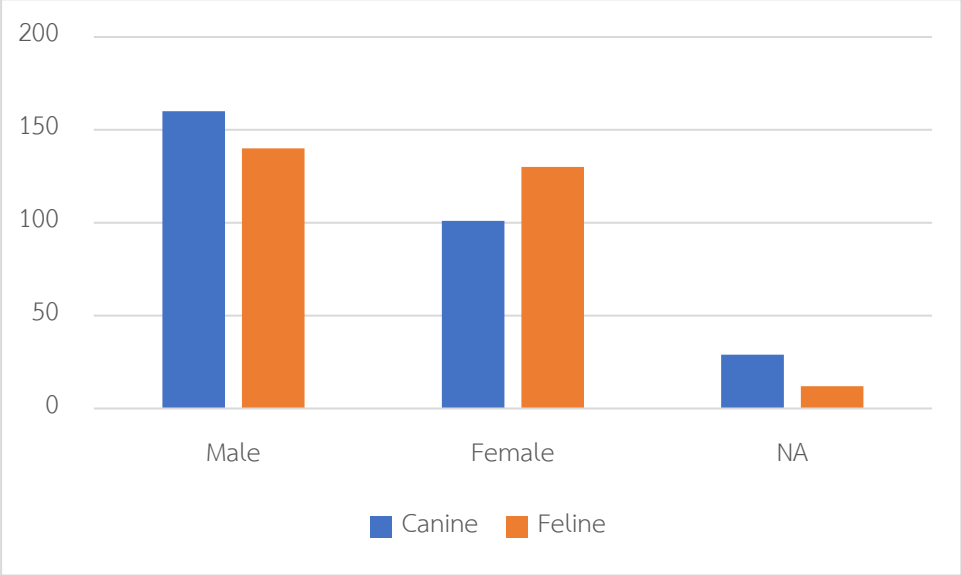


Figure 23: Bar charts of rectal swab samples collected by sex

4.2 Identification of RVA in dogs and cats by specific RT-PCR assay

In this thesis, we tested 572 rectal swab samples from dogs and cats. Our result showed that 11 out of 572 rectal swab samples (1.92%) collected from dogs and cats were positive for RVA. In detail, by species of animals, 8 out of 290 (2.75%) rectal swab samples from dogs were positive of canine rotavirus A (CRVA), and 3 out of 282 (1.06%) rectal swab samples from cats were found positive of feline rotavirus A (FRVA).

By year, from 8 dogs positive of CRVA, 5 dogs were sampled in year 2020 (2 in January and 3 in July 2020), while 3 dogs positive of CRVA were sampled in 2021 (June 2021). For 3 cats positive of FRVA, all cats were sampled in 2020 (1 in February, 1 in July, and 1 in October). By age of animals, 5 dogs were less than 6 months, and 3 dogs were more than 10 years old, while 2 cats were 1 and 3 years of age and 1 cat was less than 6 months. For sex of animals, 6 dogs were male and 2 were female, while all 3 cats were male.

It is noted that most positive samples were collected from animal hospitals in Bangkok, except only 1 dog sample was collected from animal hospital in Nonthaburi province. The detail of dogs and cats that positive for RVA are shown in Table 9.

Table 9: Dogs and cats positive of RVA

ID	Collected date	Species	Age	Sex	Province
RV24998	1/2020	Dog	2 mths	Male	Bangkok
RV25012	1/2020	Dog	3 mths	Male	Bangkok
RV25045	2/2020	Cat	3 mths	Male	Bangkok
RV25132	7/2020	Cat	3 yrs	Male	Bangkok
DC25167	7/2020	Dog	3 mths	Female	Bangkok
DC25170	7/2020	Dog	3 mths	Male	Bangkok
DC25171	7/2020	Dog	3 mths	Male	Bangkok
RV25420	10/2020	Cat	1 yr	Male	Bangkok
CV27279	6/2021	Dog	14 yrs	Male	Nonthaburi
CV27286	6/2021	Dog	15 yrs	Male	Bangkok
CV27287	6/2021	Dog	10 yrs	Female	Bangkok

4.3 Genetic characterization and genetic diversity of RVA in dogs and cats

4.3.1 Whole genome sequencing of RVA

In this thesis, from 11 positive RVA samples, 3 RNA samples including RV25012, RV25045, and DC25170 were subjected to whole genome sequencing (all 11 genes of RVA). Two RNA samples (RV25012 and DC25170) were collected from dogs in January 2020 and July 2020, while 1 RNA sample (RV25045) was collected from cat in February 2020. All 3 RNA samples were collected from male dogs and cat with 3 months of age, in Bangkok. Detail of these 3 RNA samples are shown in Table 10.

Newly designed primer sets were used for amplification of these 3 RNA samples for whole genome sequencing. The primers were designed using Primer 3 plus program. The primer sequences, size of amplified products, and PCR condition of each primer are present in Table 11 and 12.

From 3 RNA samples including RV25012 and DC25170 from dogs, and RV25045 from cat, we had accomplished the whole genome sequences of almost all samples, except VP1 gene of DC25170. The product size of each segment were 3300bp for VP1, 2700bp for VP2, 2581bp for VP3, 2300bp for VP4, 1350bp for VP6, 1036bp for VP7, 1500bp for NSP1, 1021bp for NSP2, 1100bp for NSP3, 750bp for NSP4, and 780bp for NSP5.

Table 10: Description of viruses subjected to whole genome sequencing

ID	Collected date	Species	Age	Sex	Province
RV25012	1/2020	Dog	3 mths	Male	Bangkok
RV25045	2/2020	Cat	3 mths	Male	Bangkok
DC25170	7/2020	Dog	3 mths	Male	Bangkok

Table 11: List of newly design sequencing primers used in this study

Primer name	Type	Sequence	Gene	Position (nt)	Product size(bp)	Species	Reference
RVA_VP1F_out	F	GGCTATTWAAGCTGTACAATGG	VP1	1-914	914	Dog, Cat	Charoenkul et al. 2020
RVA_VP1R_914	R	CCAGCTTTCTTCATATTATC					This study
RVA_VP1F_757	F	CCTATGTCAATTTTAGTCGC	VP1	757-1927	1170	Dog	This study
RVA_VP1R_1927	R	TTATCATCKCCATCAACTCG					
RVA_VP1F_1774	F	ATACAGTATGGAGCAGTTGC	VP1	1774-2554	780	Dog	This study
RVA_VP1R_2554	R	CTTTGCTTTYTCAGTTAGTGC					
RVA_VP1F_2036	F	GGTTTCRACGGTTGGAATAG	VP1	2036-2836	800	Dog	This study
RVA_VP1R_2836	R	GAAATTGCTGATTTAGATCC					
RVA_VP1F_2736	F	AAATTTATGCCRACTTTGCC	VP1	2736-3300	564	Dog, Cat	This study
RVA_VP1R_out	R	GGTCACATCYAAGCACTC					Charoenkul et al. 2020
RVA_VP1F_out	F	GGCTATTWAAGCTGTACAATGG	VP1	1-1333	1333	Dog	Charoenkul et al. 2020
RVA_VP1R_1333	R	TTCCATTAGCCATATCATCC					This study
RVA_VP1F_1228	F	ATGTCATCAGCATCAAATGG	VP1	1228-1927	699	Dog	This study
RVA_VP1R_1927	R	TTATCATCKCCATCAACTCG					
RVA_VP1F_757	F	CCTATGTCAATTTTAGTCGC	VP1	757-1333	576	Cat	This study
RVA_VP1R_1333	R	TTCCATTAGCCATATCATCC					
RVA_VP1F_1228	F	ATGTCATCAGCATCAAATGG	VP1	1228-2026	798	Cat	This study
RVA_VP1R_2026	R	ACTWTAGCATTCATTCGTGC					
RVA_VP1F_1774	F	ATACAGTATGGAGCAGTTGC	VP1	1774-2836	1062	Cat	This study
RVA_VP1R_2836	R	GAAATTGCTGATTTAGATCC					
RVA_VP2F_out	F	GGCTATTAAAGGYTCAATG	VP2	1-945	945	Dog	Charoenkul et al. 2020
RVA_VP2R_945	R	TTTGGCCTAATGTATCTAGC					This study

Primer name	Type	Sequence	Gene	Position (nt)	Product size(bp)	Species	Reference
RVA_VP2F_674	F	CAGGATGAGGAAACTGAAGG	VP2	674-1616	942	Dog	This study
RVA_VP2R_1616	R	TAATCYACTGGCATAGTTGG					
RVA_VP2F_1266	F	GTGGCTRTTAACGGTTATACC	VP2	1266-2159	893	Dog	This study
RVA_VP2R_2159	R	GCTATRATCACTCCCTGAGC					
RVA_VP2F_2107	F	GAACATGGARCAAATTGAACG	VP2	2107-2700	593	Dog	This study
RVA_VP2R_out	R	GGTCATATCTCCACAGTG					Charoenkul et al. 2020
RVA_VP3F_out	F	GGCTWTTAAAGCAGTATGAG	VP3	1-987	987	Dog, Cat	Charoenkul et al. 2020
RVA_VP3R_987	R	GGAGTATCAAACGGATCCCA					This study
RVA_VP3F_908	F	TCAGCACCTTCRTAYTGGAT	VP3	908-1579	671	Dog	This study
RVA_VP3R_1579	R	ATCTGTCGGTAGAAAAGTCC					
RVA_VP3F_1568	F	GACTGGACTTTTTTACCAAC	VP3	1568-2547	979	Dog, Cat	This study
RVA_VP3R_2547	R	ATATCAAACACHGTATCTCC					
RVA_VP3F_884	F	TATATGGTTGGTTCAGCTCC	VP3	884-1579	695	Cat	This study
RVA_VP3R_1579	R	ATCTGTCGGTAGAAAAGTCC					
RVA_VP3F_1877	F	TCAGGTCATGTGTAYAATGC	VP3	1877-2581	704	Dog, Cat	This study
RVA_VP3R_out	R	GGTCAMATCGTGACTAG					Charoenkul et al. 2020
RVA_VP4F_863	F	GGTGGATTAGGTTATAAATGGTCAG	VP4	863-1993	1130	Dog, Cat	This study
RVA_VP4R_1993	R	CAATGTCTGGTAATGTRTTGGAG					
RVA_VP4F_1504	F	GCARTTAGGTGAACTTAGAG	VP4	1504-2351	847	Dog, Cat	This study
RVA_VP4R_2351	R	TTGCTTACAATCTACATTGC					

Table 12: Annealing temperature and extension time for each pair of newly designed primer used in PCR reaction

Primer name	Type	Sequence	Gene	Product size (bp)	Annealing temp. (°C)	Extension time (min.)	Reference
RVA_VP1F_out	F	GGCTATTWAAGCTGTACAATGG	VP1	914	45°C	1 min	Charoenkul et al. 2020
RVA_VP1R_914	R	CCAGCTTTCTTCATATTATC					This study
RVA_VP1F_757	F	CCTATGTCAATTTTAGTCGC	VP1	1170	45°C	1.30 min	This study
RVA_VP1R_1927	R	TTATCATCKCCATCAACTCG					
RVA_VP1F_1774	F	ATACAGTATGGAGCAGTTGC	VP1	780	48°C	1 min	This study
RVA_VP1R_2554	R	CTTTGCTTTYTCAGTTAGTGC					
RVA_VP1F_2036	F	GGTTTCRACGGTTGGAATAG	VP1	800	45°C	1 min	This study
RVA_VP1R_2836	R	GAAATTGCTGATTTAGATCC					
RVA_VP1F_2736	F	AAATTTATGCCRACTTTGCC	VP1	564	48°C	1 min	This study
RVA_VP1R_out	R	GGTCACATCYAAGCACTC					Charoenkul et al. 2020
RVA_VP1F_out	F	GGCTATTWAAGCTGTACAATGG	VP1	1333	45°C	2 min	Charoenkul et al. 2020
RVA_VP1R_1333	R	TTCCATTAGCCATATCATCC					This study
RVA_VP1F_1228	F	ATGTCATCAGCATCAAATGG	VP1	699	45°C	1 min	This study
RVA_VP1R_1927	R	TTATCATCKCCATCAACTCG					
RVA_VP1F_757	F	CCTATGTCAATTTTAGTCGC	VP1	576	45°C	1 min	This study
RVA_VP1R_1333	R	TTCCATTAGCCATATCATCC					
RVA_VP1F_1228	F	ATGTCATCAGCATCAAATGG	VP1	798	45°C	1 min	This study
RVA_VP1R_2026	R	ACTWTAGCATTCATTCGTGC					
RVA_VP1F_1774	F	ATACAGTATGGAGCAGTTGC	VP1	1062	45°C	1.30 min	This study
RVA_VP1R_2836	R	GAAATTGCTGATTTAGATCC					
RVA_VP2F_out	F	GGCTATTAAAGGYTCAATG	VP2	945	45°C	1 min	Charoenkul et al. 2020
RVA_VP2R_945	R	TTTGGCCTAATGTATCTAGC					This study

Primer name	Type	Sequence	Gene	Product size (bp)	Annealing temp. (°C)	Extension time (min.)	Reference
RVA_VP2F_674	F	CAGGATGAGGAAACTGAAGG	VP2	942	48°C	1 min	This study
RVA_VP2R_1616	R	TAATCYACTGGCATAGTTGG					
RVA_VP2F_1266	F	GTGGCTRTTAACGGTTATACC	VP2	893	48°C	1 min	This study
RVA_VP2R_2159	R	GCTATRATCACTCCCTGAGC					
RVA_VP2F_2107	F	GAACATGGARCAAATTGAACG	VP2	593	48°C	1 min	This study
RVA_VP2R_out	R	GGTCATATCTCCACAGTG					Charoenkul et al. 2020
RVA_VP3F_out	F	GGCTWTTAAAGCAGTATGAG	VP3	987	45°C	1 min	Charoenkul et al. 2020
RVA_VP3R_987	R	GGAGTATCAAACGGATCCCA					This study
RVA_VP3F_908	F	TCAGCACCTTCRTAYTGGAT	VP3	671	48°C	1 min	This study
RVA_VP3R_1579	R	ATCTGTCGGTAGAAAAGTCC					
RVA_VP3F_1568	F	GACTGGACTTTTTTACCAAC	VP3	979	45°C	1 min	This study
RVA_VP3R_2547	R	ATATCAAACACHGTATCTCC					
RVA_VP3F_884	F	TATATGGTTGGTTCAGCTCC	VP3	695	48°C	1 min	This study
RVA_VP3R_1579	R	ATCTGTCGGTAGAAAAGTCC					
RVA_VP3F_1877	F	TCAGGTCATGTGTAYAATGC	VP3	704	45°C	1 min	This study
RVA_VP3R_out	R	GGTCAMATCGTGACTAG					Charoenkul et al. 2020
RVA_VP4F_863	F	GGTGGATTAGGTTATAAATGGTCAG	VP4	1130	50°C	1.30 min	This study
RVA_VP4R_1993	R	CAATGTCTGGTAATGTRTTGGAG					
RVA_VP4F_1504	F	GCARTTAGGTGAACTTAGAG	VP4	847	45°C	1 min	This study
RVA_VP4R_2351	R	TTGCTTACAATCTACATTGC					

4.3.2 Genetic diversity of RVA by genotyping of rotavirus A viruses

Genotyping of rotavirus A viruses in this study by Virus pathogen resource (ViPR) web software showed that all 3 viruses (RV25012, RV25045, DC25170) were classified into genotype G3P[3] according to the analysis of VP7 and VP4 gene sequences. Based on the classification of all 11 genes (VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5), our results showed that 2 viruses from dogs (RV25012, DC25170) were classified into genotype G3-P[3]-I3-R3-C3-M3-A9-N2-T3-E3-H6 which are the same as previous report in Thailand (Charoenkul et al., 2020). On the other hand, the virus from cat (RV25045) was classified into genotype G3-P[3]-I8-R3-C3-M3-A9-N3-T3-E3-H6 which never been reported in Thailand before. Moreover, according to the report from all over the world, the genotype I8 of VP6 gene has never been reported in cats before

(Table 13 shows genotypes of RVA based on whole genome sequence analysis).

Table 13: Genotype of all 11 segments of RVA with complete genome sequencing in this study

Virus	Strain	Species	Year	Country	Gene										
					VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
Canine															
RVA/Dog/THA/RV25012/2020/G3P[3]	RV25012	Dog	2020	THA	G3	P[3]	I3	R3	C3	M3	A9	N2	T3	E3	H6
RVA/Dog/THA/DC25170/2020/G3P[3]	DC25170	Dog	2020	THA	G3	P[3]	I3	NA	C3	M3	A9	N2	T3	E3	H6
Feline															
RVA/Cat/THA/RV25045/2020/G3P[3]	RV25045	Cat	2020	THA	G3	P[3]	I8	R3	C3	M3	A9	N3	T3	E3	H6

4.3.3 Genetic diversity of RVA by phylogenetic analysis

Phylogenetic analysis of RVA was performed by the comparison of each gene segments of the RVAs from this study with those of reference RVAs in the GenBank databases. The reference RVAs were selected including the viruses from different species, different geographic regions, and different time of isolation.

For phylogenetic analysis of VP7 gene, 2 viruses from dogs in this study (RV25012 and DC25170) were closely related to RVA viruses from dogs in Thailand previously reported in 2017 and 2019 (Charoenkul et al., 2020). The viruses were clustered together with almost other reference RVA strain of dogs, which are the genotype G3, subcluster b2. It is interesting to noted that there are some human's RVA belonged to subcluster b2, for example strain 12638 reported in Japan which was closely related to dog's RVA viruses in this study (Okitsu et al., 2018). Another example, human's RVA strain HCR3A found in USA and strain Ro1845 found in Israel were belong to subcluster b2 (Tsugawa and Hoshino, 2008). On the other hands, the virus from cat in this study (RV25045) clustered with the viruses of subcluster b1 which clustering together with many bat and human RVA strains. Our analysis showed that cat RVA strain FRV348 (Nakagomi et al., 2018) and dog RVA strain RV52-96 (Matthijnssens et al., 2011b) were also clustered in this subcluster b1. It is interesting to noted that RV25045 was the most closely related to human's virus strain CMH222 and CMH079 found in Chiangmai province, Thailand in 2001 (Khamrin et al., 2006) and 2005 (Khamrin et al., 2009), respectively (Figure 24 shows the phylogenetic tree of VP7 gene).

For phylogenetic of VP4 gene, similar to VP7 gene, 2 viruses from dogs in this study (RV25012 and DC25170) were closely related to dog's RVA viruses from previous report in Thailand (Charoenkul et al., 2020), and clustered together with other reference RVA strains from dogs, as well as bat's RVA strain LUS12-14 found in Zambia (Sasaki et al., 2016). While virus from cat in this study (RV25045) was closely related to human's virus strain CMH222 reported in Chiangmai province, Thailand in 2001 (Khamrin et al., 2006) and bat's RVA strain MSLH14 and LZHP2 in China (He et al., 2013) (He et al., 2017) (Figure 25 shows the phylogenetic tree of VP4 gene).

For phylogenetic analysis of VP6 gene, 2 viruses from dogs in this study (RV25012 and DC25170) were closely related to human's RVA strain 12638 reported in Japan (Okitsu et al., 2018) and dog's RVA from previous report in Thailand (Charoenkul et al., 2020). In this cluster, we also noted that one cat's RVA strain FRV348 found in Japan (Nakagomi et al., 2018), unusual human's RVA strain PA260-97 (Matthijnssens et al., 2011b), strain HCR3A and strain Ro1845 (Tsugawa and Hoshino, 2008) were grouped into this cluster. While virus from cat in this study (RV25045) was closely related to human's RVA strain CMH079 (Khamrin et al., 2009) and recent human's RVA strain MS2015-1-0001 found in Tak province, Thailand (Komoto et al., 2021) as well as bat's RVA strain MYAS33 from China (Xia et al., 2014). We also found that another 2 bat's RVA strain BSTM70 and MSLH14 (He et al., 2013) along with human's virus strain CMH222 (Khamrin et al., 2006) were clustered within the same cluster (I8 cluster) (Figure 26 shows the phylogenetic tree of VP6 gene).

For phylogenetic analysis of VP1 gene, virus from dog in this study (RV25012) was closely related to dog's RVA from previous report in Thailand (Charoenkul et al., 2020) and unusual human's RVA strain 12638 (Okitsu et al., 2018). While virus from cat in this study (RV25045) was closely related to bat's RVA strain MYAS33 in China (Xia et al., 2014) and human's RVA strain MS2015-1-0001 (Komoto et al., 2021), as well as RVA found in simian, USA (Brown et al., 2011) (Figure 27 shows the phylogenetic tree of VP1 gene).

For phylogenetic analysis of VP2 gene, 2 viruses from dogs in this study (RV25012 and DC25170) were closely related to dog's RVA from previous report in Thailand (Charoenkul et al., 2020), and human's RVA strain 12638 (Okitsu et al., 2018) and strain PA260-97 (Matthijnssens et al., 2011b), as well as cat's virus strain FRV348 (Nakagomi et al., 2018). While virus from cat in this study (RV25045) was closely related to RVA found in simian, USA (Brown et al., 2011) (Figure 28 shows the phylogenetic tree of VP2 gene).

For phylogenetic analysis of VP3 gene, 2 viruses from dogs in this study (RV25012 and DC25170) were closely related to dog's RVA from previous report in Thailand (Charoenkul et al., 2020) and bat's RVA strain LUS12-14 reported in Zambia (Sasaki et al., 2016). While virus from cat in this study (RV25045) was closely related

to human's RVA strain MS2015-1-0001 (Komoto et al., 2021), and bat's RVA found in China, strain MYAS33 (Xia et al., 2014), and strain MSLH14 (He et al., 2013) (Figure 29 shows the phylogenetic tree of VP3 gene).

For phylogenetic analysis of NSP1 gene, 2 viruses from dogs in this study (RV25012 and DC25170) were closely related to dog's RVA from previous report in Thailand (Charoenkul et al., 2020) and human's RVA strain 12638 reported in Japan (Okitsu et al., 2018), as well as bat's RVA strain LUS12-14 reported in Zambia (Sasaki et al., 2016). While virus from cat in this study (RV25045) was closely related to bat's RVA strain MYAS33 (Xia et al., 2014) and strain MSLH14 (He et al., 2013) from China. This cat's virus also clustered with human's RVA strain MS2015-1-0001 (Komoto et al., 2021) and simian's RVA found in USA (Brown et al., 2011) (Figure 30 shows the phylogenetic tree of NSP1 gene).

For phylogenetic analysis of NSP2 gene, 2 viruses from dogs in this study (RV25012 and DC25170) were closely related to dog's RVA from previous report in Thailand (Charoenkul et al., 2020) and unusual human's RVA, strain 12638 from Japan (Okitsu et al., 2018) and strain PA260-97 from Italy (Matthijnssens et al., 2011b), as well as bat's RVA strain LUS12-14 from Zambia (Sasaki et al., 2016). While virus from cat in this study (RV25045) was closely related to human's RVA strain MS2015-1-0001 found in Thailand (Komoto et al., 2021) (Figure 31 shows the phylogenetic tree of NSP2 gene).

For phylogenetic analysis of NSP3 gene, 2 viruses from dogs in this study (RV25012 and DC25170) were closely related to cat's reference RVA strain FRV348 (Nakagomi et al., 2018) and dog's RVA from previous report in Thailand (Charoenkul et al., 2020) and bat's RVA strain LUS12-14 from Zambia (Sasaki et al., 2016), as well as human's RVA strain 12638 (Okitsu et al., 2018). While virus from cat in this study (RV25045) was closely related to bat's RVA strain MYAS333 (Xia et al., 2014) and strain LZHP2 (He et al., 2017) as well as simian's RVA found in USA (Brown et al., 2011) (Figure 32 shows the phylogenetic tree of NSP3 gene).

For phylogenetic analysis of NSP4 gene, 2 viruses from dogs in this study (RV25012 and DC25170) were closely related to dog's RVA from previous report in Thailand (Charoenkul et al., 2020) and cat's reference RVA strain FRV348 (Nakagomi

et al., 2018), as well as unusual human's RVA strain PA260-97 (Matthijnssens et al., 2011b) and strain 12638 (Okitsu et al., 2018). While virus from cat in this study (RV25045) was closely related to human's RVA strain MS2015-1-0001 (Komoto et al., 2021) and bat's RVA strain MSLH14 (He et al., 2013) (Figure 33 shows the phylogenetic tree of NSP4 gene).

For phylogenetic analysis of NSP5 gene, 2 viruses from dogs in this study (RV25012 and DC25170) were closely related to dog's RVA from previous report in Thailand (Charoenkul et al., 2020) and human's RVA strain 12638 (Okitsu et al., 2018). While virus from cat in this study was closely related to bat's RVA strain MSLH14 (He et al., 2013) and human's RVA strain MS2015-1-0001 (Komoto et al., 2021) as well as bat's RVA strain MYAS33 (Xia et al., 2014) and strain LZHP2 (He et al., 2017) (Figure 34 shows the phylogenetic tree of NSP5 gene).

In conclusion, our phylogenetic analysis showed that genetic composition of 2 RVA from dogs in this study (RV25012 and DC25170) had closest genetic relationship with RVA in dogs from the previous report in Thailand (Charoenkul et al., 2020) at 9 gene segment (VP1, VP2, VP3, VP4, VP7, NSP1, NSP2, NSP4, NSP5), excepting in VP6 gene that had closest genetic relationship with human's RVA strain 12638 found in Japan (Okitsu et al., 2018) and NSP3 gene segment that had closest genetic relationship with cat's reference RVA strain FRV348 (Nakagomi et al., 2018). While genetic composition (VP7 and VP4 genes) of RVA from cat in this study (RV25045) had closest genetic relationship with human's RVA strain CMH222 found in Chiangmai province, Thailand, in 2001 (Khamrin et al., 2006) and VP7 and VP6 genes had closest genetic relationship with human's RVA strain CMH079 found in Chiangmai province, Thailand, in 2005 (Khamrin et al., 2009). Moreover VP3, NSP2, and NSP4 gene of cat's RVA in this study had closest genetic relationship with human's RVA strain MS2015-1-0001 found in Tak province, Thailand (Komoto et al., 2021). Interestingly, some gene of cat's RVA in this study also had close genetic relationship with bat's RVA from China, including strain MYAS33 (VP1, NSP1, and NSP3) (Xia et al., 2014), strain MSLH14 (VP6, VP3, and NSP4) (He et al., 2013), strain LZHP2 (VP4, NSP3, and NSP4 genes) (He et al., 2017) as well as strain BSTM70 (VP6) (He et al., 2013).

Moreover, VP2 gene (and VP1, NSP1, and NSP3 genes) of cat's RVA in this study was closely related to simian's RVA from USA (Brown et al., 2011).

The summarize of RVA strains closest related to viruses in this study in each segment showed in Table 14 for dog and Table 15 for cat.

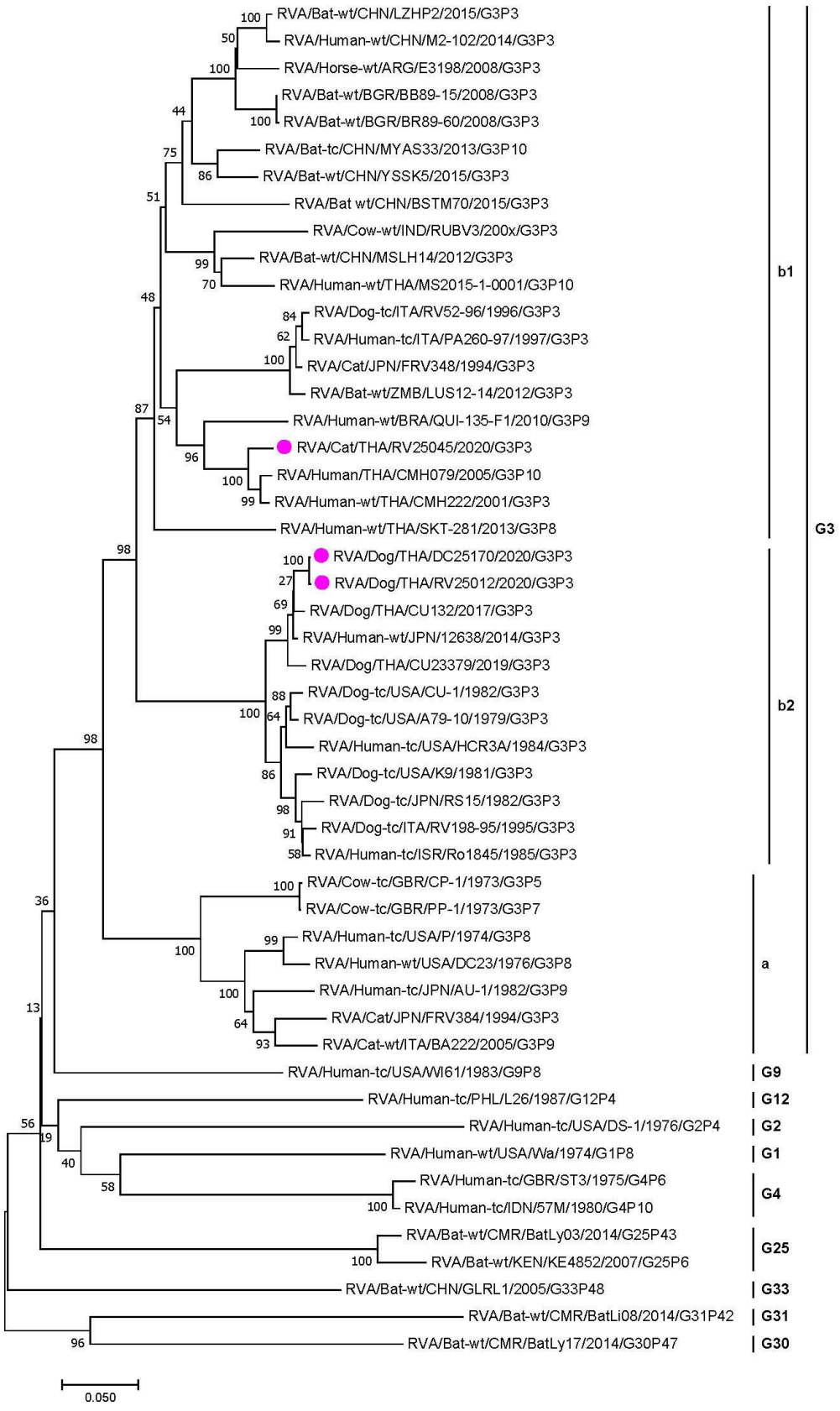


Figure 24: Phylogenetic tree of VP7 gene

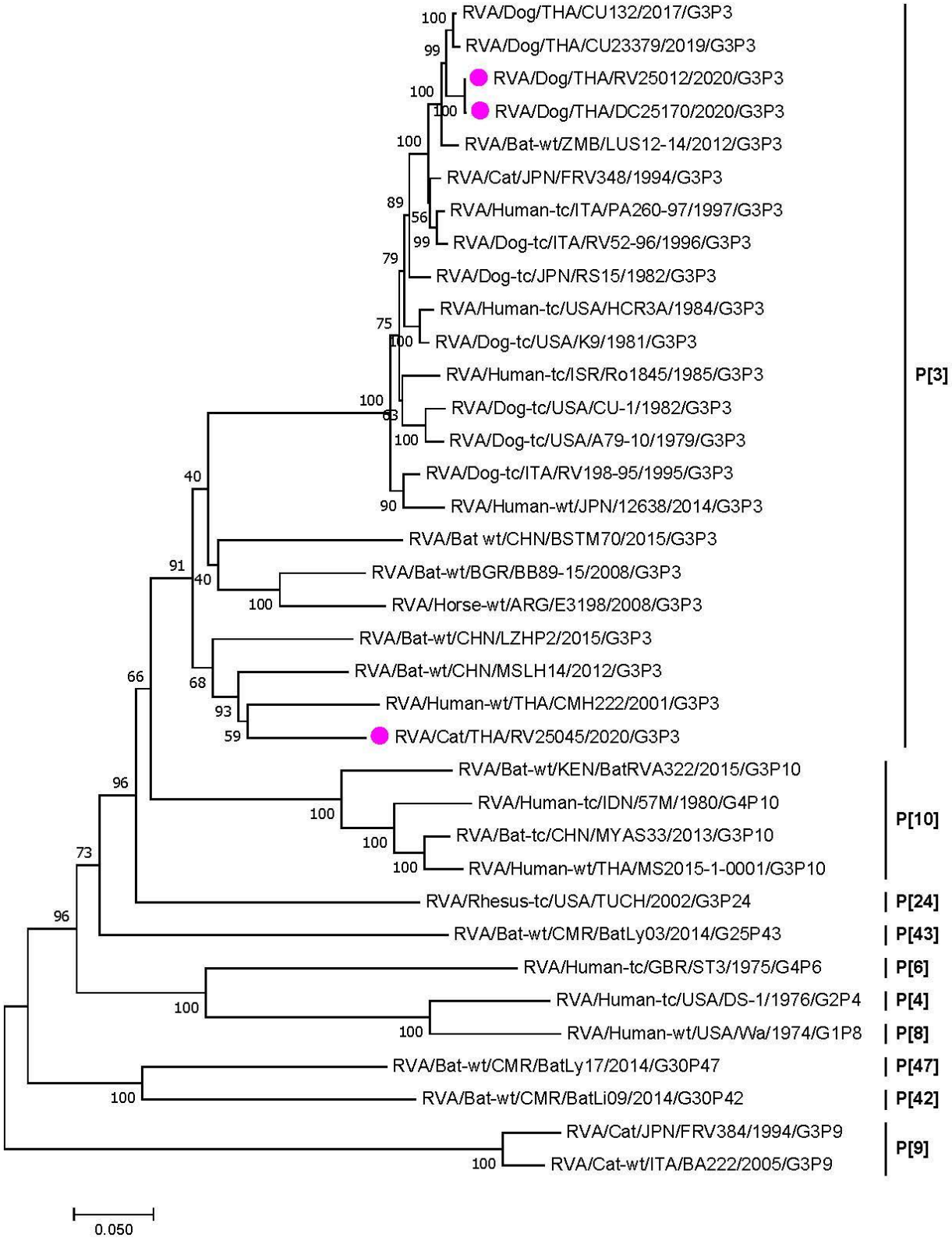


Figure 25: Phylogenetic tree of VP4 gene

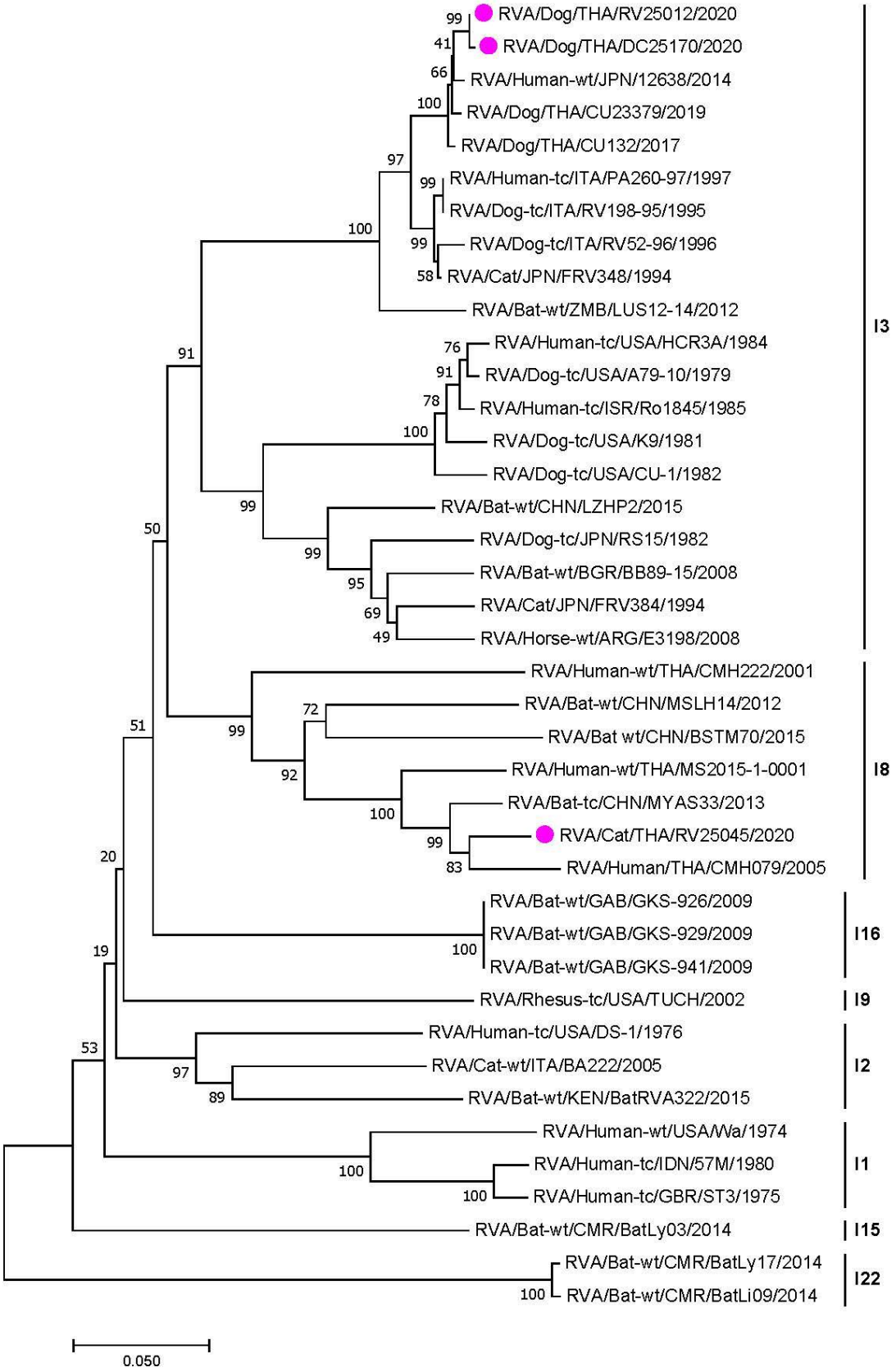


Figure 26: Phylogenetic tree of VP6 gene

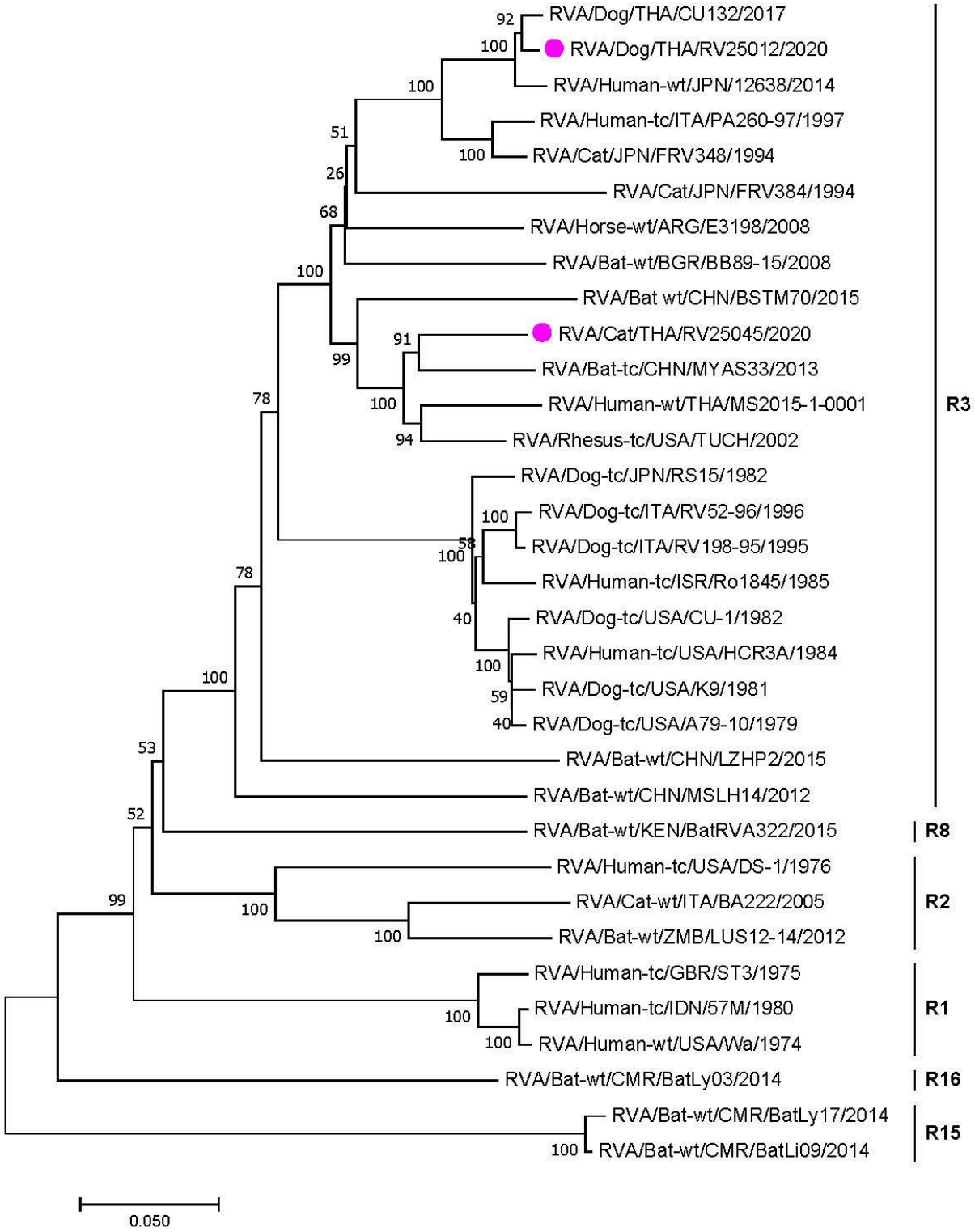


Figure 27: Phylogenetic tree of VP1 gene

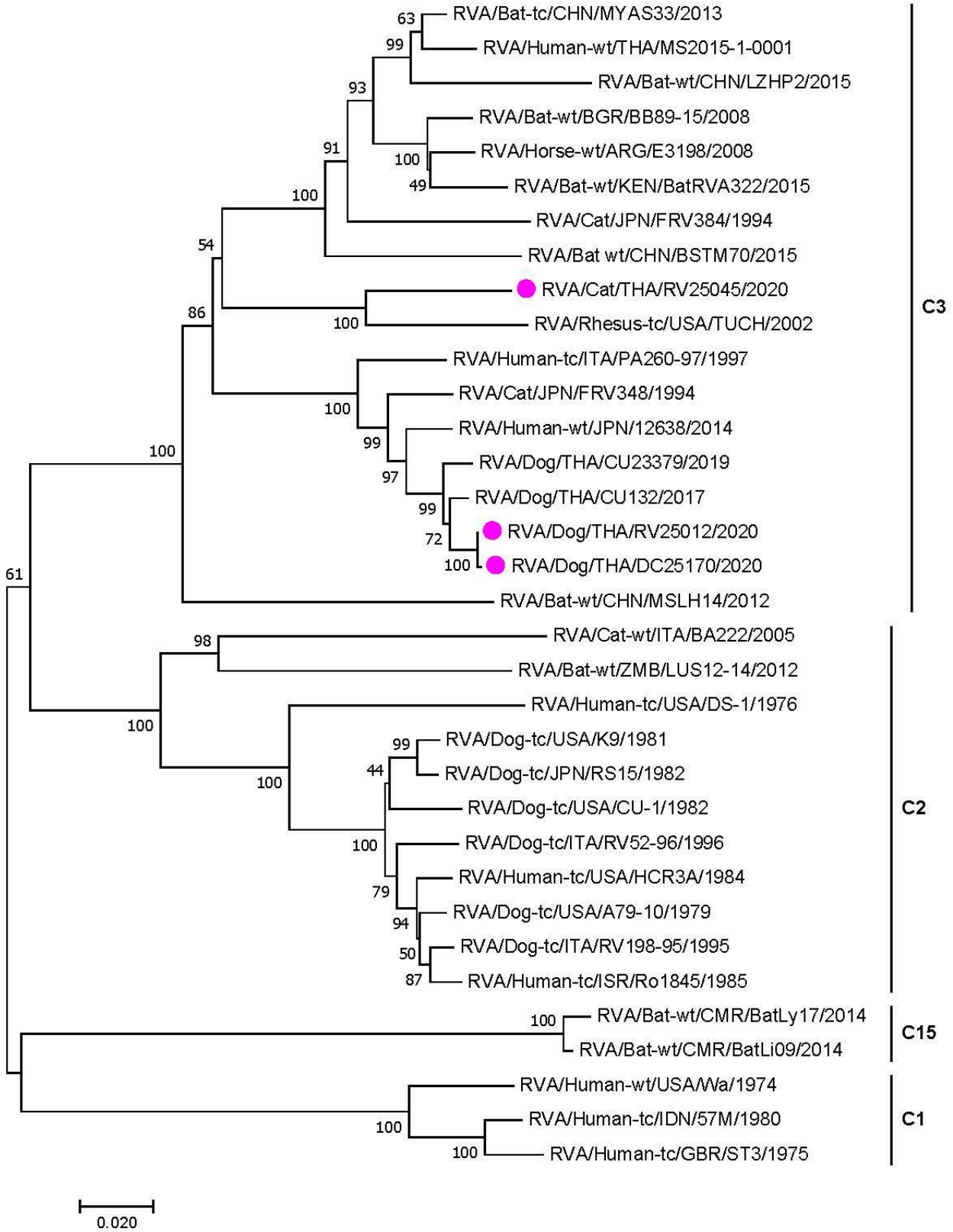


Figure 28: Phylogenetic tree of VP2 gene

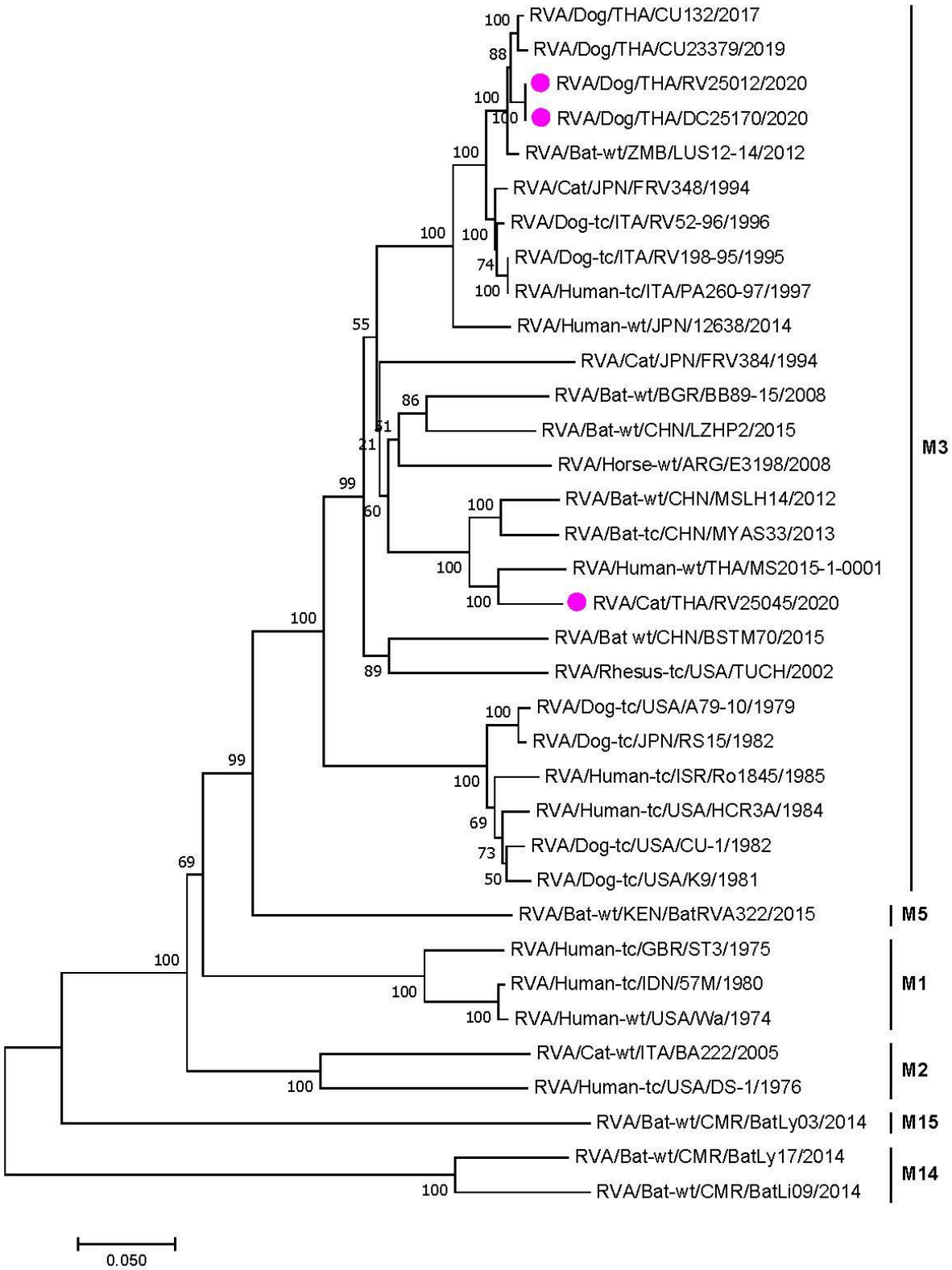


Figure 29: Phylogenetic tree of VP3 gene

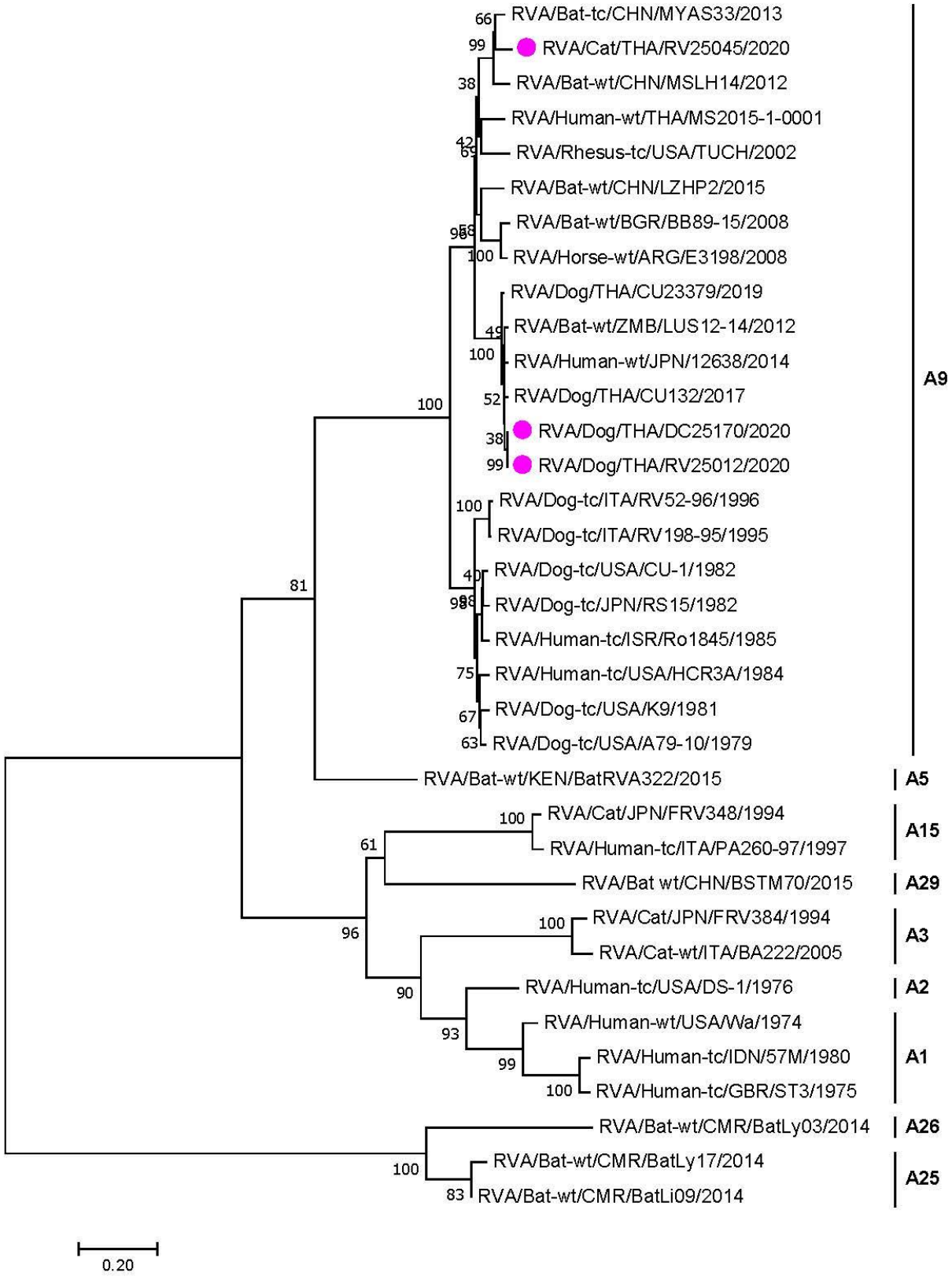


Figure 30: Phylogenetic tree of NSP1 gene

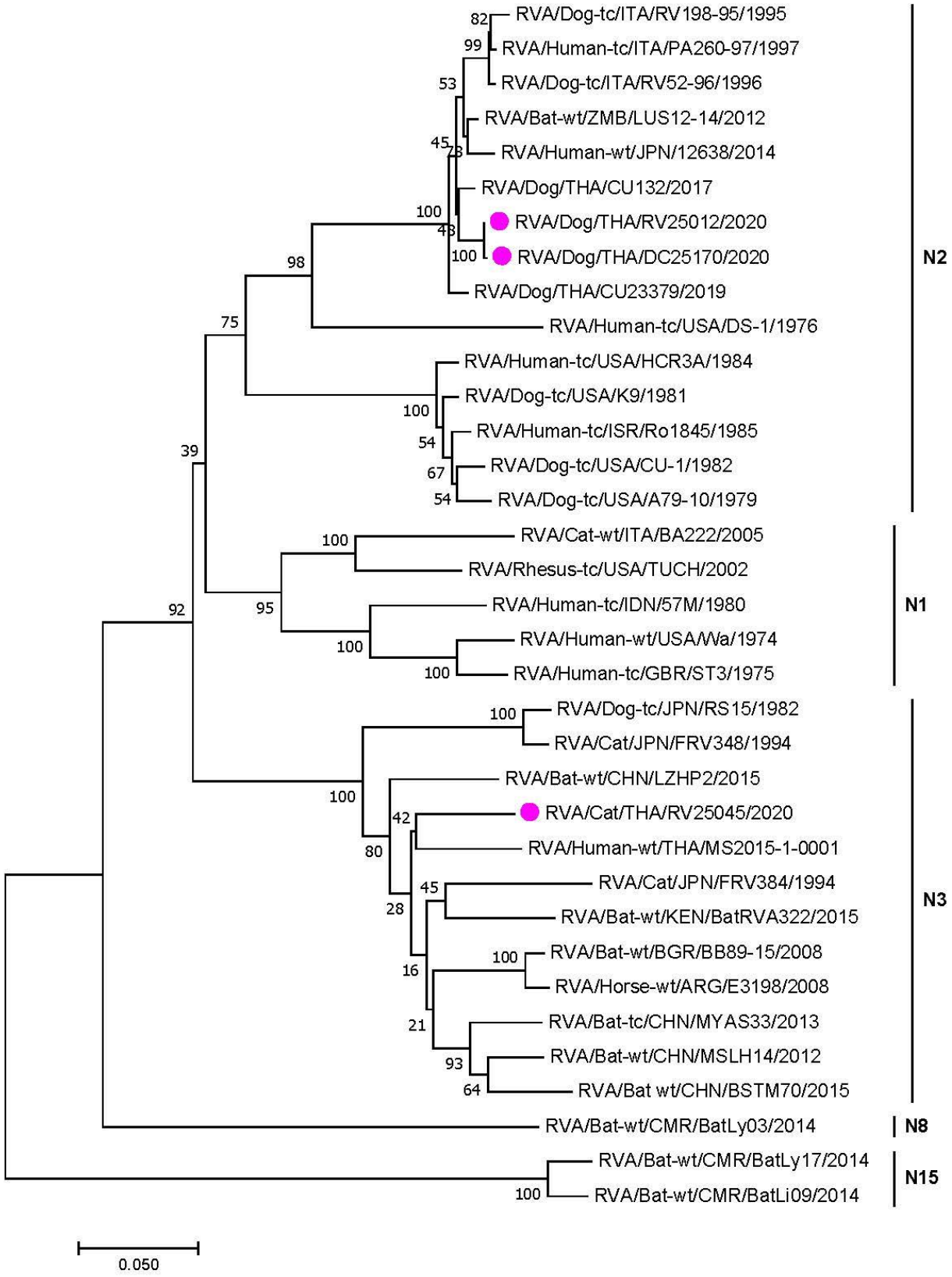


Figure 31: Phylogenetic tree of NSP2 gene

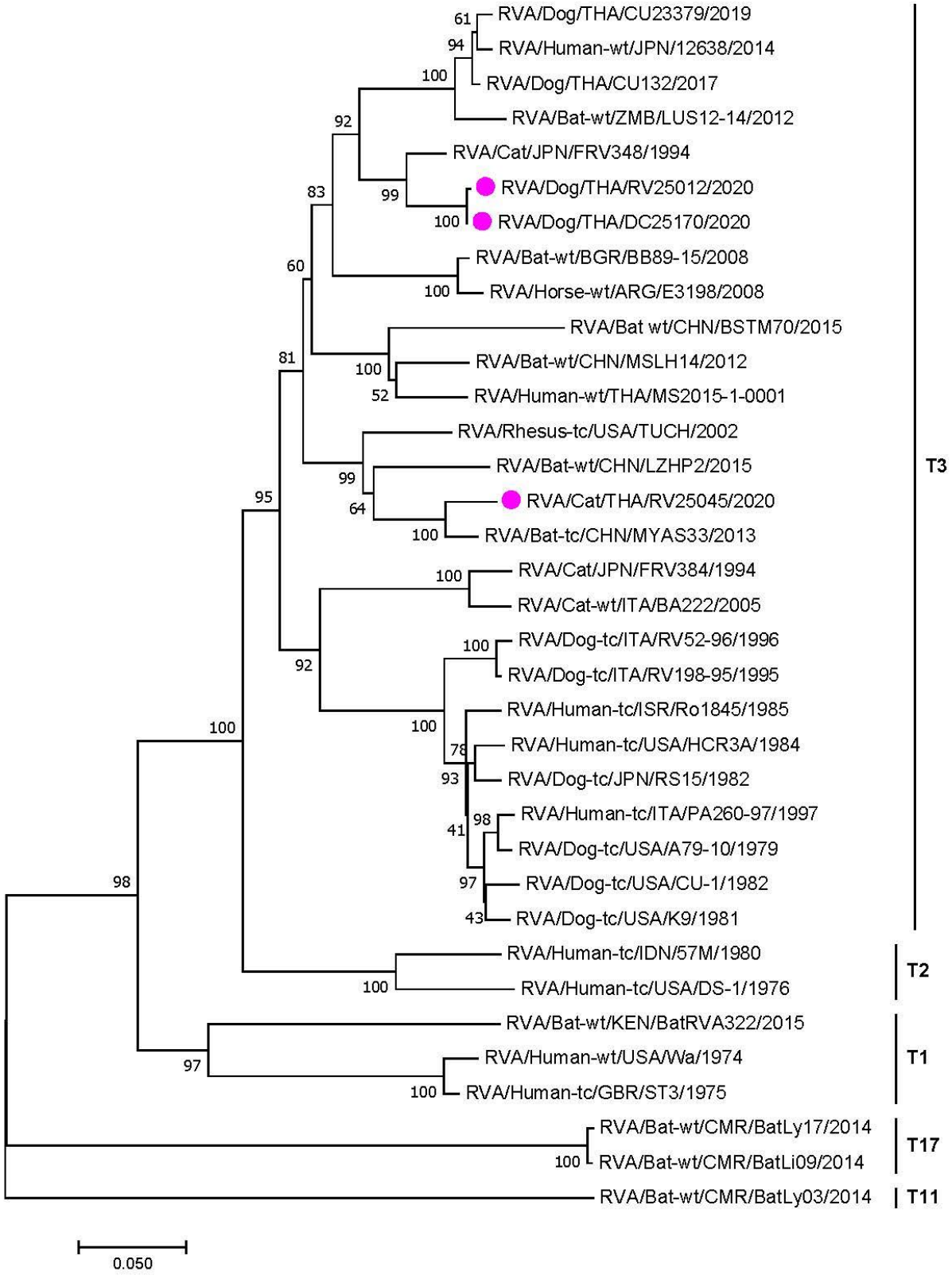


Figure 32: Phylogenetic tree of NSP3 gene

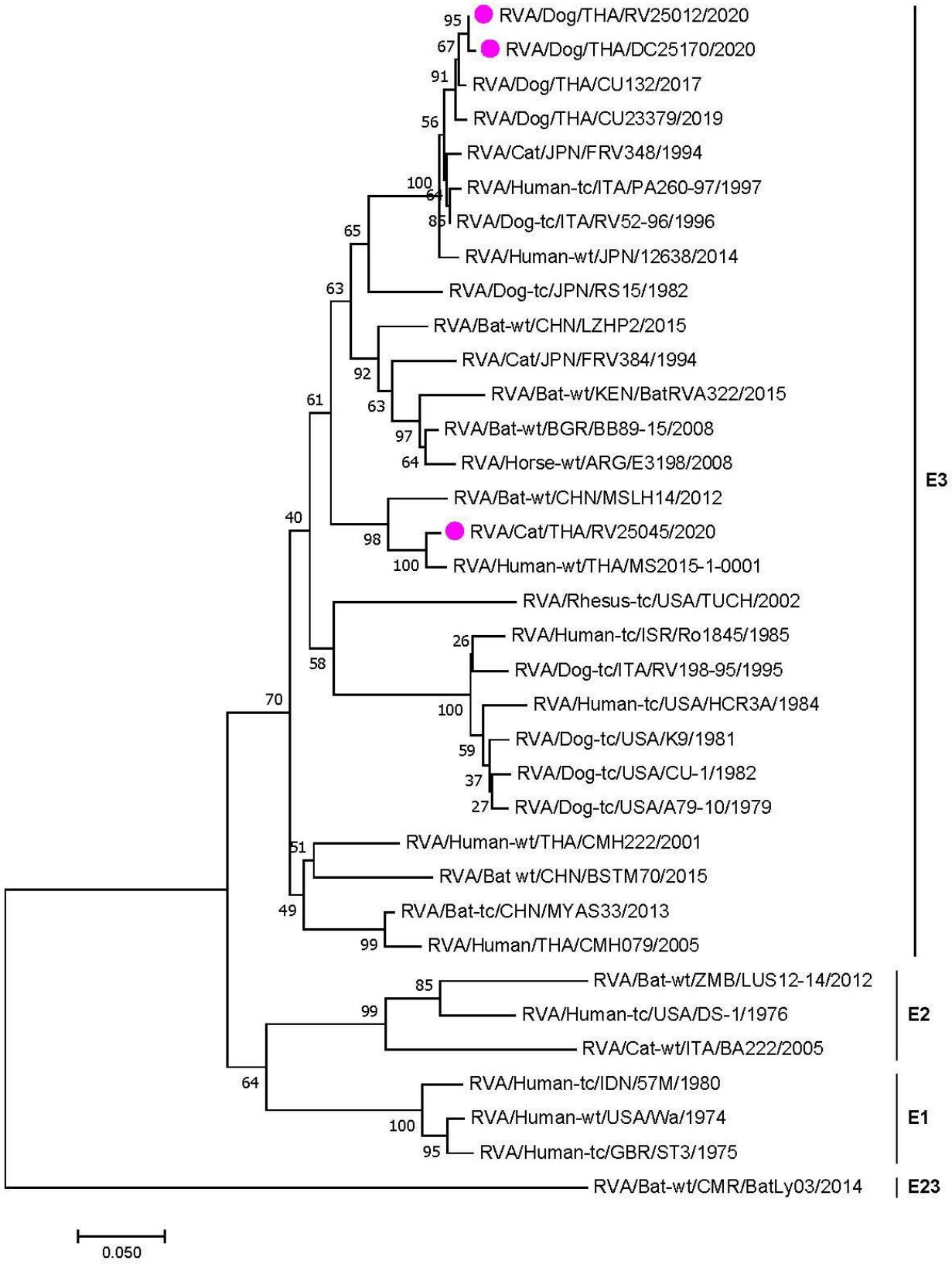


Figure 33: Phylogenetic tree of NSP4 gene

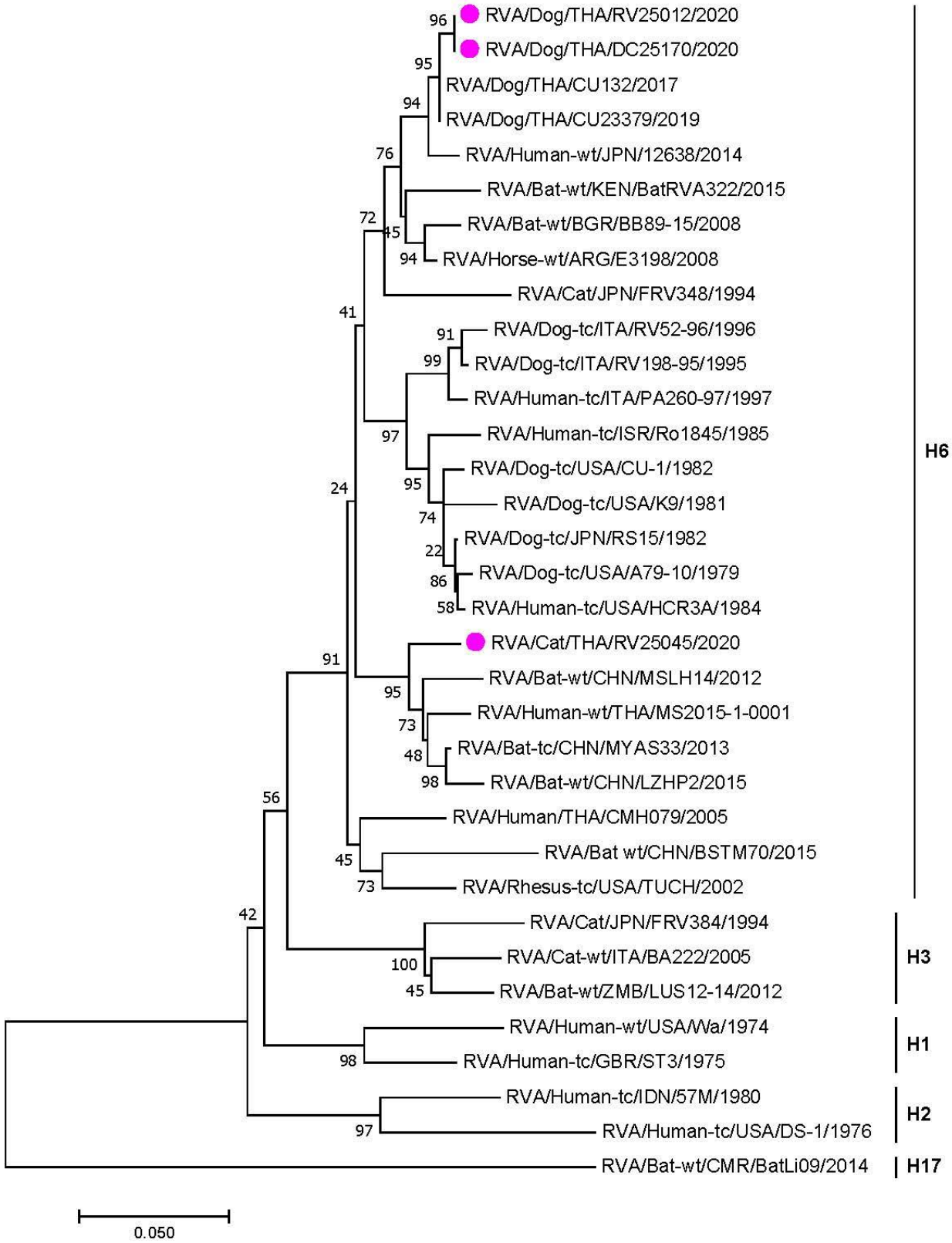


Figure 34: Phylogenetic tree of NSP5 gene

Table 14: Summarize of RVA strains closest related to dog's virus in this study (RV25012, DC25170) by BLASTn analysis

Gene	Strain	Species	Year	Country
VP7	RVA/Dog/THA/CU132/2017/G3P[3]	Dog	2017	THA
VP4	RVA/Dog/THA/CU132/2017/G3P[3]	Dog	2017	THA
VP6	RVA/Human-wt/JPN/12638/2014/G3P[3]	Human	2014	JPN
VP1	RVA/Dog/THA/CU132/2017/G3P[3]	Dog	2017	THA
VP2	RVA/Dog/THA/CU132/2017/G3P[3]	Dog	2017	THA
VP3	RVA/Dog/THA/CU132/2017/G3P[3]	Dog	2017	THA
NSP1	RVA/Dog/THA/CU132/2017/G3P[3]	Dog	2017	THA
NSP2	RVA/Dog/THA/CU132/2017/G3P[3]	Dog	2017	THA
NSP3	RVA/Cat/JPN/FRV348/1994/G3P[3]	Cat	1994	JPN
NSP4	RVA/Dog/THA/CU132/2017/G3P[3]	Dog	2017	THA
NSP5	RVA/Dog/THA/CU132/2017/G3P[3]	Dog	2017	THA

Table 15: Summarize of RVA strains closest related to cat's virus in this study (RV25045) by BLASTn analysis

Gene	Strain	Species	Year	Country
VP7	RVA/Human-wt/THA/CMH222/2001/G3P[3]	Human	2001	THA
	RVA/Human-wt/THA/CMH079/2005/G3P[10]	Human	2005	THA
VP4	RVA/Human-wt/THA/CMH222/2001/G3P[3]	Human	2001	THA
VP6	RVA/Human-wt/THA/CMH079/2005/G3P[10]	Human	2005	THA
VP1	RVA/Bat-wt/CHN/MYAS33/2012/G3P[3]	Bat	2013	CHN
VP2	RVA/Rhesus-tc/USA/TUCH/2002/G3P24	Simian	2002	USA
VP3	RVA/Human-wt/THA/MS2015-1-0001/G3P10	Human	2015	THA
NSP1	RVA/Bat-wt/CHN/MYAS33/2012/G3P[3]	Bat	2013	CHN
NSP2	RVA/Human-wt/THA/MS2015-1-0001/G3P10	Human	2015	THA
NSP3	RVA/Bat-wt/CHN/MYAS33/2012/G3P[3]	Bat	2013	CHN
NSP4	RVA/Human-wt/THA/MS2015-1-0001/G3P10	Human	2015	THA
NSP5	RVA/Bat-wt/CHN/MSLH14/2012/G3P[3]	Bat	2012	CHN

4.3.4 Genetic diversity of RVA by nucleotide and amino acid identities

Nucleotide and amino acid identities of RVA was performed by the comparison of each gene segments of the RVA in this study with those of closely related strains, as well as some other reference RVA strains with similar genotype from the GenBank databases. For dog's RVA, RV25012 was selected as the representative of this study to compare with other strains. For cat, RV25045 was the representative of cat's RVA in this study. The percentage of nucleotide (nt) and amino acid (aa) identities between RVA in this study and those reference strains are showed in Table 16 and Table 17 for dog, and Table 18 and Table 19 for cat.

For nucleotide and amino acid identities of VP7 gene, dog's RVA in this study (RV25012) had closest nucleotide identity with dog's RVA strain CU132 from previous reported in Thailand, 2017 (Charoenkul et al., 2020) and human's RVA strain 12638 found in Japan, 2014 (Okitsu et al., 2018), which were 98.26%, 98.56% (nt identities), respectively, and 99.38% and 99.38% (aa identities). Almost other dog's reference RVA strains also had close identity with dog's virus in this study, which approximately around 93-95% (nt), and 98% (aa). It is noted that there were other 2 unusual human's RVA that had close identity with dog's RVA in this study, which were strain HCR3A found in USA and strain Ro1845 found in Israel (Tsugawa and Hoshino, 2008), which were 94.97%, 94.26% (nt) and 99.07%, 98.75% (aa), respectively. On the other hands, the virus from cat in this study (RV25045) had closest identity with human's RVA strain CMH079 found in Chiangmai province, Thailand, 2005 (Khamrin et al., 2009), with 97.23% (nt) and 99.07% (aa).

For nucleotide and amino acid identities of VP4 gene, dog's RVA in this study (RV25012) had closest identity with dog's RVA strain CU132 from previous reported in Thailand, 2017 (Charoenkul et al., 2020), which was 98.37% (nt) and 99.09% (aa). It is noted that, there were other 2 RVA strains that had close identity with dog's RVA in this study, which were bat's RVA strain LUS12-14 found in Zambia (Sasaki et al., 2016) and cat's RVA strain FRV348 found in Japan (Nakagomi et al., 2018), with 97.51%, 97.16% (nt) and 98.43%, 97.76% (aa), respectively. While virus from cat in this study (RV25045) had closest identity with human's virus strain CMH222 reported in

Chiangmai province, Thailand in 2001 (Khamrin et al., 2006) and bat's RVA strain MSLH14 in China (He et al., 2013), with 87.66%, 87.61% (nt) and 95.79%, 97.60% (aa), respectively.

For nucleotide and amino acid identities of VP6 gene, dog's RVA in this study (RV25012) had closest identity with dog's RVA strain CU23379 from previous report in Thailand (Charoenkul et al., 2020) and human's RVA strain 12638 reported in Japan (Okitsu et al., 2018), with 99.05% and 99.05% (nt), and 99.48% and 99.48% (aa). It is noted that there were some others RVA strain that had close identity with dog's RVA in this study, which were one cat's RVA strain FRV348 found in Japan (Nakagomi et al., 2018), two dog's RVA strain RV198-95 and strain RV52-96, as well as unusual human's RVA strain PA260-97 (Matthijnssens et al., 2011b), with approximately around 96% (nt), and 98-99% (aa). While virus from cat in this study (RV25045) had closest identity with bat's RVA strain MYAS33 from China (Xia et al., 2014), with 95.84% (nt) and 100% (aa). It is noted that there were other 2 human's RVA reported in Thailand that had close identity with cat's RVA in this study, which were strain CMH079, with 94.89% (nt) and 98.95% (aa), and strain MS2015-1-0001, with 93.47% (nt) and 100% (aa).

For nucleotide and amino acid identities of VP1 gene, dog's RVA in this study (RV25012) had closest identity with dog's RVA strain CU132 from previous report in Thailand (Charoenkul et al., 2020), with 98.72% (nt) and 99.45% (aa). It also had close identity with unusual human's RVA strain 12638 (Okitsu et al., 2018), with 98.05% (nt) and 99.63% (aa). While virus from cat in this study (RV25045) had closest identity with simian's RVA reported in USA (Brown et al., 2011), with 93.17% (nt) and 97.84% (aa). It also had close identity with bat's RVA strain MYAS33 in China (Xia et al., 2014), with 93.02% (nt) and 98.10% (aa), as well as human's RVA strain MS2015-1-0001 (Komoto et al., 2021), with 92.10% (nt) and 98.13% (aa).

For nucleotide and amino acid identities of VP2 gene, dog's RVA in this study (RV25012) had closest identity with dog's RVA strain CU132 and CU23379 from previous report in Thailand (Charoenkul et al., 2020), with 98.75%, 98.28% (nt) and 99.65%, 99.88% (aa), respectively. It is noted that it also had close identity with human's RVA strain 12638 (Okitsu et al., 2018) with 97.07% (nt) and 99.76% (aa), and

strain PA260-97 (Matthijnssens et al., 2011b) with 95.08% (nt) and 99.53% (aa), as well as cat's virus strain FRV348 (Nakagomi et al., 2018) with 96.21% (nt) and 99.65% (aa). While virus from cat in this study (RV25045) had closest identity with simian's RVA reported in USA (Brown et al., 2011) with 92.83% (nt) and 98.63% (aa).

For nucleotide and amino acid identities of VP3 gene, dog's RVA in this study (RV25012) had closest identity with dog's RVA strain CU132 and CU23379 from previous report in Thailand (Charoenkul et al., 2020), with 98.68%, 98.37% (nt) and 99.03%, 99.28% (aa), respectively. It is noted that one bat's RVA strain LUS12-14 reported in Zambia (Sasaki et al., 2016) also had close identity with dog's RVA in this study, with 98.52% (nt) and 99.03% (aa). While virus from cat in this study (RV25045) had closest identity with human's RVA strain MS2015-1-0001 (Komoto et al., 2021), with 94.06% (nt) and 97.07% (aa). It is noted that two bat's RVA from China, which were strain MYAS33 (Xia et al., 2014) and strain MSLH14 (He et al., 2013) also had close identity with cat's RVA in this study, with 92.03%, 92.07% (nt) and 96.40%, 96.66% (aa), respectively.

For nucleotide and amino acid identities of NSP1 gene, dog's RVA in this study (RV25012) had closest identity with dog's RVA strain CU132 from previous report in Thailand (Charoenkul et al., 2020), with 98.57% (nt) and 99.38% (aa). It also had close identity with human's RVA strain 12638 reported in Japan (Okitsu et al., 2018), as well as bat's RVA strain LUS12-14 reported in Zambia (Sasaki et al., 2016), with 98.23%, 98.16% (nt) and 98.54%, 98.75% (aa), respectively. While virus from cat in this study (RV25045) had closest identity with bat's RVA strain MYAS33 from China (Xia et al., 2014), with 94.04% (nt) and 90.24% (aa). It also had close identity with bat's RVA strain MSLH14 from China (He et al., 2013), with 92.38% (nt) and 88.59% (aa).

For nucleotide and amino acid identities of NSP2 gene, dog's RVA in this study (RV25012) had closest identity with dog's RVA strain CU132 from previous report in Thailand (Charoenkul et al., 2020), with 98.32% (nt) and 99.37% (aa). It also had close identity with two unusual human's RVA strain 12638 from Japan (Okitsu et al., 2018) and strain PA260-97 from Italy (Matthijnssens et al., 2011b) with 97.48%, 97.17% (nt) and 98.40%, 99.04% (aa), respectively, as well as bat's RVA strain LUS12-

14 from Zambia (Sasaki et al., 2016) with 97.90% (nt) and 98.40% (aa). While virus from cat in this study (RV25045) had closest identity with human's RVA strain MS2015-1-0001 found in Thailand (Komoto et al., 2021) with 92.66% (nt) and 99.04% (aa).

For nucleotide and amino acid identities of NSP3 gene, interestingly, dog's RVA in this study (RV25012) had closest identity with cat's reference RVA strain FRV348 found in Japan, 1994 (Nakagomi et al., 2018) with 95.65% (nt) and 98.05% (aa). While virus from cat in this study (RV25045) had closest identity with bat's RVA strain MYAS333 found in China (Xia et al., 2014) with 96.07% (nt) and 97.38% (aa).

For nucleotide and amino acid identities of NSP4 gene, dog's RVA in this study (RV25012) had closest identity with dog's RVA strain CU132 from previous report in Thailand (Charoenkul et al., 2020), with 99.05% (nt) and 98.84% (aa). It also had close identity with two unusual human's RVA strain 12638 from Japan (Okitsu et al., 2018) and strain PA260-97 from Italy (Matthijnssens et al., 2011b) with 97.16%, 97.73% (nt), respectively, and 98.84% equally (aa), as well as cat's reference RVA strain FRV348 (Nakagomi et al., 2018) with 97.73% (nt) and 97.66% (aa). While virus from cat in this study (RV25045) had closest identity with human's RVA strain MS2015-1-0001 (Komoto et al., 2021) with 98.11% (nt) and 98.84% (aa). It also had close identity with bat's RVA strain MSLH14 (He et al., 2013) with 94.32% (nt) and 98.26% (aa).

For nucleotide and amino acid identities of NSP5 gene, dog's RVA in this study (RV25012) had closest identity with dog's RVA strain CU132 and CU23379 from previous report in Thailand (Charoenkul et al., 2020), with 99.50%, 99.33% (nt) and 98.98%, 98.98% (aa), respectively. It also had close identity with human's RVA strain 12638 found in Japan (Okitsu et al., 2018) with 98.16% (nt) and 98.46% (aa). While virus from cat in this study (RV25045) had closest identity with human's RVA strain MS2015-1-0001 (Komoto et al., 2021) and bat's RVA strain MYAS33 (Xia et al., 2014) with 96.82% (nt) and 98.46% (aa), equally. It also had close identity with bat's RVA strain MSLH14 (He et al., 2013) with 96.15% (nt) and 97.94% (aa).

In conclusion, the pairwise comparison of nucleotide and amino acid identity showed that, dog's RVA in this study (RV25012) had closest identity with dog's RVA

from the previous report in Thailand (Charoenkul et al., 2020) in 10 gene segments (VP1, VP2, VP3, VP4, VP6, VP7, NSP1, NSP2, NSP4, NSP5), but in VP7 and VP6 gene also closest to human's RVA strain 12638 found in Japan (Okitsu et al., 2018). Interestingly, the only exception was NSP3 gene that had closest identity with cat's reference RVA strain FRV348 (Nakagomi et al., 2018). On the other hands, cat's RVA in this study had closest identity with human's RVA strain CMH079 (Khamrin et al., 2009) in VP7 gene, and strain CMH222 (Khamrin et al., 2006) in VP4 gene, as well as strain MS2015-1-0001 (Komoto et al., 2021) in VP3, NSP2, NSP4, NSP5 genes, these human's RVA were all found in Thailand. There were bat's RVA found in China that had closest identity with cat's RVA in this study, which were strain MSLH14 (He et al., 2013) in VP4 gene, and strain MYAS33 (Xia et al., 2014) in VP6, NSP1, NSP3, NSP5 gene. In VP1 and VP2 gene, cat's RVA in this study had closest identity with simian's RVA reported in USA (Brown et al., 2011).

Table 16: Nucleotide (nt) and amino acid (aa) identities between dog's RVA in this study (RV25012) and reference RVA strains

Virus	Strain	Species	Year	Country	Nucleotide (nt) and Amino acid (aa) identities (%)											
					Gene											
					VP7		VP4		VP6		VP1		VP2		VP3	
					nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
RVA/Dog/THA/RV25012/2020/G3P[3]	**RV25012**	**Dog**	**2020**	**THA**												
RVA/Dog/THA/DC25170/2020/G3P[3]	**DC25170**	**Dog**	**2020**	**THA**	99.90	100.00	99.91	100.00	99.83	99.74	NA	NA	99.88	100.00	99.96	100.00
RVA/Dog-tc/USA/A79-10/1979/G3P[3]	A79-10	Dog	1979	USA	95.59	98.75	93.77	97.35	84.76	96.50	86.12	95.40	82.08	96.72	84.93	91.28
RVA/Dog-tc/USA/K9/1981/G3P[3]	K9	Dog	1981	USA	94.46	98.44	95.10	96.67	84.33	96.50	85.58	95.00	82.04	96.72	85.01	91.70
RVA/Dog-tc/USA/CU-1/1982/G3P[3]	CU-1	Dog	1982	USA	95.59	98.75	93.43	96.40	84.59	96.77	86.12	95.20	81.80	96.46	85.29	91.42
RVA/Dog-tc/JPN/RS15/1982/G3P[3]	RS15	Dog	1982	JPN	93.74	98.75	95.40	97.22	85.19	97.87	86.12	95.40	82.23	96.72	85.09	91.56
RVA/Dog-tc/ITA/RV198-95/1995/G3P[3]	RV198-95	Dog	1995	ITA	94.05	98.75	94.12	96.67	96.97	99.48	86.00	95.20	81.88	96.84	97.05	97.92
RVA/Dog-tc/ITA/RV52-96/1996/G3P[3]	RV52-96	Dog	1996	ITA	84.31	93.79	96.65	97.08	96.10	98.95	86.00	95.20	82.27	96.21	97.17	98.29
RVA/Dog/THA/CU132/2017/G3P[3]	CU132	Dog	2017	THA	98.26	99.38	98.37	99.09	98.96	99.74	98.72	99.45	98.75	99.65	98.68	99.03
RVA/Dog/THA/CU23379/2019/G3P[3]	CU23379	Dog	2019	THA	97.33	98.75	98.11	98.96	99.05	99.48	98.48	99.26	98.28	99.88	98.37	99.28
RVA/Cat-tc/AUS/Cat97/1984/G3P[3]	Cat97	Cat	1984	AUS	94.05	98.75	93.99	96.67	84.68	96.50	85.94	94.80	81.61	96.59	84.73	91.84
RVA/Cat/JPN/FRV348/1994/G3P[3]	FRV348	Cat	1994	JPN	84.31	94.48	97.16	97.76	96.97	99.48	93.61	97.56	96.21	99.65	97.21	98.54
RVA/Bat-wt/ZMB/LUS12-14/2012/G3P[3]	LUS12-14	Bat	2012	ZMB	84.00	93.79	97.51	98.43	94.46	99.21	80.10	93.57	81.69	96.97	98.52	99.03
RVA/Bat-wt/CHN/MSLH14/2012/G3P[3]	MSLH14	Bat	2012	CHN	85.64	95.16	81.62	91.47	82.08	96.50	84.54	95.80	87.54	99.53	87.24	94.17
RVA/Bat-wt/CHN/MYAS33/2012/G3P[10]	MYAS33	Bat	2013	CHN	85.64	95.83	75.77	84.52	82.51	96.77	87.40	96.20	88.52	99.41	86.80	93.77
RVA/Bat-wt/CHN/LZHP2/2015/G3P[3]	LZHP2	Bat	2015	CHN	84.92	94.82	81.01	87.21	85.37	97.33	85.15	93.15	87.04	95.96	87.32	90.99
RVA/Human-tc/USA/HCR3A/1984/G3P[3]	HCR3A	Human	1984	USA	94.97	99.07	94.80	96.53	84.50	96.77	85.64	95.40	81.96	96.84	85.17	91.84
RVA/Human-tc/ISR/Ro1845/1985/G3P[3]	Ro1845	Human	1985	ISR	94.26	98.75	93.73	96.67	84.94	96.77	85.51	95.40	81.84	96.97	84.61	90.99
RVA/Human-tc/ITA/PA260-97/1997/G3P[3]	PA260-97	Human	1997	ITA	83.90	93.79	96.82	97.49	96.97	99.48	93.73	97.94	95.08	99.53	97.05	97.92
RVA/Human-wt/JPN/12638/2014/G3P[3]	12638	Human	2014	JPN	98.56	99.38	92.91	96.26	99.05	99.48	98.05	99.63	97.07	99.76	94.06	97.42

Table 17: Nucleotide (nt) and amino acid (aa) identities between dog's RVA in this study (RV25012) and reference RVA strains (cont.)

Virus	Strain	Species	Year	Country	Nucleotide (nt) and Amino acid (aa) identities (%)									
					Gene									
					NSP1		NSP2		NSP3		NSP4		NSP5	
					nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
RVA/Dog/THA/RV25012/2020/G3P[3]	**RV25012**	**Dog**	**2020**	**THA**										
RVA/Dog/THA/DC25170/2020/G3P[3]	**DC25170**	**Dog**	**2020**	**THA**	99.73	99.79	99.90	100.00	99.89	100.00	99.62	100.00	100.00	100.00
RVA/Dog-tc/USA/A79-10/1979/G3P[3]	A79-10	Dog	1979	USA	83.41	85.41	84.91	92.54	85.77	92.07	84.28	92.64	93.97	95.24
RVA/Dog-tc/USA/K9/1981/G3P[3]	K9	Dog	1981	USA	82.73	84.60	85.74	92.54	85.56	92.07	84.28	92.64	93.47	94.12
RVA/Dog-tc/USA/CU-1/1982/G3P[3]	CU-1	Dog	1982	USA	82.87	85.14	85.53	92.91	85.46	91.32	84.28	93.29	94.64	95.24
RVA/Dog-tc/JPN/RS15/1982/G3P[3]	RS15	Dog	1982	JPN	82.73	84.60	80.71	86.79	86.52	92.81	91.86	95.21	94.81	95.24
RVA/Dog-tc/ITA/RV198-95/1995/G3P[3]	RV198-95	Dog	1995	ITA	83.14	85.14	96.65	98.40	86.84	92.07	84.09	92.64	94.30	94.68
RVA/Dog-tc/ITA/RV52-96/1996/G3P[3]	RV52-96	Dog	1996	ITA	82.66	84.32	97.27	98.72	86.41	92.07	98.30	98.84	93.63	94.68
RVA/Dog/THA/CU132/2017/G3P[3]	CU132	Dog	2017	THA	98.57	99.38	98.32	99.37	90.98	95.32	99.05	98.84	99.50	98.98
RVA/Dog/THA/CU23379/2019/G3P[3]	CU23379	Dog	2019	THA	98.09	98.96	97.80	99.68	90.98	94.97	98.67	98.84	99.33	98.98
RVA/Cat-tc/AUS/Cat97/1984/G3P[3]	Cat97	Cat	1984	AUS	82.94	84.32	85.32	92.54	86.41	92.44	84.28	92.64	93.63	93.55
RVA/Cat/JPN/FRV348/1994/G3P[3]	FRV348	Cat	1994	JPN	43.28	-152.90	80.82	86.79	95.65	98.05	97.73	97.66	94.14	95.79
RVA/Bat-wt/ZMB/LUS12-14/2012/G3P[3]	LUS12-14	Bat	2012	ZMB	98.16	98.75	97.90	98.40	90.13	94.97	78.03	80.14	89.28	91.80
RVA/Bat-wt/CHN/MSLH14/2012/G3P[3]	MSLH14	Bat	2012	CHN	86.69	92.26	80.29	87.19	87.37	92.81	88.45	93.94	93.30	96.88
RVA/Bat-wt/CHN/MYAS33/2012/G3P[10]	MYAS33	Bat	2013	CHN	87.44	92.73	80.61	87.59	86.62	94.61	87.12	93.94	93.97	97.41
RVA/Bat-wt/CHN/LZHP2/2015/G3P[3]	LZHP2	Bat	2015	CHN	87.03	90.56	81.24	86.79	85.56	92.81	91.29	94.58	92.96	95.79
RVA/Human-tc/USA/HCR3A/1984/G3P[3]	HCR3A	Human	1984	USA	82.73	84.60	85.74	92.91	85.56	92.44	83.71	92.64	94.30	94.68
RVA/Human-tc/ISR/Ro1845/1985/G3P[3]	Ro1845	Human	1985	ISR	82.87	84.60	85.64	92.91	86.09	93.17	84.47	92.64	93.80	93.55
RVA/Human-tc/ITA/PA260-97/1997/G3P[3]	PA260-97	Human	1997	ITA	43.07	-150.36	97.17	99.04	85.67	91.70	97.73	98.84	94.30	95.24
RVA/Human-wt/JPN/12638/2014/G3P[3]	12638	Human	2014	JPN	98.23	98.54	97.48	98.40	90.76	95.32	97.16	98.84	98.16	98.46

Table 18: Nucleotide (nt) and amino acid (aa) identities between cat's RVA in this study (RV25045) and reference RVA strains

Virus	Strain	Species	Year	Country	Nucleotide (nt) and Amino acid (aa) identities (%)											
					Gene											
					VP7		VP4		VP6		VP1		VP2		VP3	
					nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
RVA/Cat/THA/RV25045/2020/G3P[3]	**RV25045**	**Cat**	**2020**	**THA**												
RVA/Cat-tc/AUS/Cat97/1984/G3P[3]	Cat97	Cat	1984	AUS	85.44	95.83	81.05	91.21	82.51	95.93	86.02	96.22	81.25	95.72	82.81	91.70
RVA/Cat-tc/AUS/Cat2/1984/G3P[9]	Cat2	Cat	1984	AUS	82.36	92.03	67.71	58.75	81.21	97.33	85.95	96.02	81.48	95.84	82.62	90.99
RVA/Cat/JPN/FRV348/1994//G3P[3]	FRV348	Cat	1994	JPN	88.10	96.50	81.10	91.36	81.99	96.77	88.79	97.41	88.12	98.14	87.64	93.90
RVA/Cat/JPN/FRV384/1994//G3P[3]	FRV384	Cat	1994	JPN	81.44	92.03	67.54	58.75	80.87	97.05	87.04	96.52	87.48	98.38	85.73	92.54
RVA/Cat-wt/ITA/BA222/2005/G3P[9]	BA222	Cat	2005	ITA	81.44	91.30	67.62	59.27	81.21	95.37	79.73	93.64	80.65	95.10	76.20	81.73
RVA/Bat-wt/CHN/MSLH14/2012/G3P[3]	MSLH14	Bat	2012	CHN	88.72	97.48	87.61	97.60	87.88	99.48	84.05	96.62	86.34	98.38	92.07	96.66
RVA/Bat-wt/CHN/MYAS33/2012/G3P[3]	MYAS33	Bat	2013	CHN	89.13	98.12	77.71	87.70	95.84	100.00	93.02	98.10	88.65	98.38	92.03	96.40
RVA/Human-wt/THA/CMH222/2001/G3P[3]	CMH222	Human	2001	THA	97.03	98.75	87.66	95.79	83.29	98.14	NA	NA	NA	NA	NA	NA
RVA/Human-wt/THA/CMH079/2005/G3P[10]	CMH079	Human	2005	THA	97.23	99.07	NA	NA	94.89	98.95	NA	NA	NA	NA	NA	NA
RVA/Human-tc/THA/CU-365/2008/G3P[9]	CU365	Human	2008	THA	82.15	92.03	67.54	59.52	81.73	97.60	88.10	96.52	87.21	98.38	87.24	94.04
RVA/Human-wt/JPN/12638/2014/G3P[3]	12638	Human	2014	JPN	84.62	94.48	81.44	90.74	81.13	96.77	88.10	97.21	88.61	98.38	87.60	94.17
RVA/Human-wt/THA/MS2015-1-0001/G3P10	MS2015-1-0001	Human	2015	THA	88.38	97.16	77.52	87.54	93.47	100.00	92.10	98.13	88.40	97.93	94.06	97.04
RVA/Rhesus-tc/USA/TUCH/2002/G3P24	TUCH	Simian	2002	USA	79.10	87.97	76.75	85.71	81.32	95.53	93.17	97.84	92.83	98.63	85.77	92.95

Table 19: Nucleotide (nt) and amino acid (aa) identities between cat's RVA in this study (RV25045) and reference RVA strains (cont.)

Virus	Strain	Species	Year	Country	Nucleotide (nt) and Amino acid (aa) identities (%)									
					Gene									
					NSP1		NSP2		NSP3		NSP4		NSP5	
					nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
RVA/Cat/THA/RV25045/2020/G3P[3]	**RV25045**	**Cat**	**2020**	**THA**										
RVA/Cat-tc/AUS/Cat97/1984/G3P[3]	Cat97	Cat	1984	AUS	82.67	58.94	79.56	87.59	84.29	93.90	84.47	93.94	93.47	95.24
RVA/Cat-tc/AUS/Cat2/1984/G3P[9]	Cat2	Cat	1984	AUS	50.59	NA	80.61	89.16	76.75	79.62	85.23	95.21	89.78	93.55
RVA/Cat/JPN/FRV348/1994//G3P[3]	FRV348	Cat	1994	JPN	51.21	NA	88.68	97.41	87.58	96.70	88.83	97.06	92.63	96.34
RVA/Cat/JPN/FRV384/1994//G3P[3]	FRV384	Cat	1994	JPN	50.87	NA	91.51	96.74	85.46	95.67	89.39	98.84	88.44	91.21
RVA/Cat-wt/ITA/BA222/2005/G3P[9]	BA222	Cat	2005	ITA	50.31	NA	79.66	85.97	85.88	95.67	78.22	84.11	88.94	91.80
RVA/Bat-wt/CHN/MSLH14/2012/G3P[3]	MSLH14	Bat	2012	CHN	92.38	88.59	91.19	98.40	86.31	94.61	94.32	98.26	96.15	97.94
RVA/Bat-wt/CHN/MYAS33/2012/G3P[3]	MYAS33	Bat	2013	CHN	94.04	90.24	90.78	98.40	96.07	97.38	90.53	98.26	96.82	98.46
RVA/Human-wt/THA/CMH222/2001/G3P[3]	CMH222	Human	2001	THA	NA	NA	NA	NA	NA	NA	87.69	99.43	NA	NA
RVA/Human-wt/THA/CMH079/2005/G3P[10]	CMH079	Human	2005	THA	NA	NA	NA	NA	NA	NA	89.58	97.06	93.80	97.41
RVA/Human-tc/THA/CU-365/2008/G3P[9]	CU365	Human	2008	THA	49.69	NA	90.99	95.72	89.07	98.38	88.64	96.45	93.63	96.88
RVA/Human-wt/JPN/12638/2014/G3P[3]	12638	Human	2014	JPN	86.63	74.66	81.55	87.59	86.20	95.67	89.02	97.06	93.80	98.46
RVA/Human-wt/THA/MS2015-1-0001/G3P[10]	MS2015-1-0001	Human	2015	THA	88.64	92.58	92.66	99.04	87.26	96.36	98.11	98.84	96.82	98.46
RVA/Rhesus-tc/USA/TUCH/2002/G3P[24]	TUCH	Simian	2002	USA	86.68	92.11	81.45	88.38	92.25	95.67	84.28	93.94	94.30	98.98

4.3.5 Genetic diversity of RVA based on genetic constellation analysis

From genotyping of each gene segment of RVA, the combination of 11 genotypes from each gene segment were analyzed and called as genetic constellation. The genetic constellation of RVA in this study were compared with closely related strains from phylogenetic analysis, as well as some other important reference strains.

The result showed that, 2 viruses from dogs in this study (RV25012 and DC25170) which had the G3-P[3]-I3-R3-C3-M3-A9-N2-T3-E3-H6 genotype, were in the same genetic constellation as dog's RVA from previous reported in Thailand, in 2017 and 2019 (Charoenkul et al., 2020). From our knowledge, this genetic constellation has never been reported in dogs in other countries before. Interestingly, there was a reported of one human's RVA that belonged in this genetic constellation, which was strain 12638 found in Japan (Okitsu et al., 2018).

On the other hands, virus from cat in this study (RV25045) which had the G3-P[3]-I8-R3-C3-M3-A9-N3-T3-E3-H6 genotype, to our knowledge, this genetic constellation has never been reported in cat before, but had been found in bat's RVA, strain MSLH14 reported in China (He et al., 2013). The genetic constellation of RVA in this study and reference strains are showed in Table 20.

Table 20: Genetic constellation of RVAs in this study and reference strains

Virus	Strain	Species	Year	Country	Gene										
					VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
This study															
Canine															
RVA/Dog/THA/RV25012/2020/G3P[3]	**RV25012**	**Dog**	**2020**	**THA**	**G3**	**P[3]**	**I3**	**R3**	**C3**	**M3**	**A9**	**N2**	**T3**	**E3**	**H6**
RVA/Dog/THA/DC25170/2020/G3P[3]	**DC25170**	**Dog**	**2020**	**THA**	**G3**	**P[3]**	**I3**	**X**	**C3**	**M3**	**A9**	**N2**	**T3**	**E3**	**H6**
Feline															
RVA/Cat/THA/RV25045/2020/G3P[3]	**RV25045**	**Cat**	**2020**	**THA**	**G3**	**P[3]**	**I8**	**R3**	**C3**	**M3**	**A9**	**N3**	**T3**	**E3**	**H6**
Reference strain															
Canine															
RVA/Dog-tc/USA/A79-10/1979/G3P[3]	A79-10	Dog	1979	USA	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Dog-tc/USA/K9/1981/G3P[3]	K9	Dog	1981	USA	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Dog-tc/USA/CU-1/1982/G3P[3]	CU-1	Dog	1982	USA	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Dog-tc/JPN/RS15/1982/G3P[3]	RS15	Dog	1982	JPN	G3	P[3]	I3	R3	C2	M3	A9	N3	T3	E3	H6
RVA/Dog-tc/ITA/RV198-95/1995/G3P3	RV198-95	Dog	1995	ITA	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Dog-tc/ITA/RV52-96/1996/G3P[3	RV52-96	Dog	1996	ITA	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Dog/THA/CU132/2017/G3P[3]	CU132	Dog	2017	THA	G3	P[3]	I3	R3	C3	M3	A9	N2	T3	E3	H6
RVA/Dog/THA/CU20139/2017/G3P[3]	CU20139	Dog	2017	THA	G3	P[3]	I3	R3	C3	M3	A9	N2	T3	E3	H6
RVA/Dog/THA/CU23379/2019/G3P[3]	CU23379	Dog	2019	THA	G3	P[3]	I3	R3	C3	M3	A9	N2	T3	E3	H6

Virus	Strain	Species	Year	Country	Gene										
					VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
Feline															
RVA/Cat-tc/AUS/Cat97/1984/G3P[3]	Cat97	Cat	1984	AUS	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Cat-tc/AUS/Cat2/1984/G3P[9]	Cat2	Cat	1984	AUS	G3	P[9]	I3	R3	C3	M3	A3	N1	T6	E3	H3
RVA/Cat/JPN/FRV348/1994//G3P[3]	FRV348	Cat	1994	JPN	G3	P[3]	I3	R3	C3	M3	A15	N3	T3	E3	H6
RVA/Cat/JPN/FRV384/1994//G3P[3]	FRV384	Cat	1994	JPN	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
RVA/Cat-wt/ITA/BA222/2005/G3P[9]	BA222	Cat	2005	ITA	G3	P[9]	I2	R2	C2	M2	A3	N1	T3	E2	H3
Horse															
RVA/Horse-wt/ARG/E30/1993/G3P[12]	E30	Horse	1993	ARG	G3	P[12]	I6	R2	C2	M3	A10	N2	T3	E12	H7
RVA/Horse/ARG/E3198/2008/G3P[3]	E3198	Horse	2008	ARG	G3	P[3]	I3	R3	C3	M3	A9	N3	T3	E3	H6
Bat															
RVA/Bat-wt/ZMB/LUS12-14/2012/G3P[3]	LUS12-14	Bat	2012	ZMB	G3	P[3]	I3	R2	C2	M3	A9	N2	T3	E2	H3
RVA/Bat-wt/CHN/MSLH14/2012/G3P[3]	MSLH14	Bat	2012	CHN	G3	P[3]	I8	R3	C3	M3	A9	N3	T3	E3	H6
RVA/Bat-wt/CHN/MYAS33/2012/G3P[10]	MYAS33	Bat	2013	CHN	G3	P[10]	I8	R3	C3	M3	A9	N3	T3	E3	H6
RVA/Bat-wt/CMR/BatLi09/2014/G30P[42]	BatLi09	Bat	2014	CMR	G30	P[42]	I22	R15	C15	M14	A25	N15	T17	E22	H17
RVA/Bat-wt/CHN/LZHP2/2015/G3P[3]	LZHP2	Bat	2015	CHN	G3	P[3]	I3	R3	C3	M3	A9	N3	T3	E3	H6
Cow															
RVA/Cow-tc/USA/WC3/1981/G6P[5]	WC3	Bovine	1981	USA	G6	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3

Virus	Strain	Species	Year	Country	Gene										
					VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
Avian															
RVA/Avian/JPN/PO13/1988/G18P[17]	PO13	Avian	1988	JPN	G18	P[17]	I4	R4	C4	N4	A4	N4	T4	E4	H4
Swine															
RVA/Pig-tc/MEX/YM/1983/G11P[7]	YM	Porcine	1983	MEX	G11	P[7]	I5	R1	C1	M1	A8	N1	T1	E1	H1
RVA/Pig-tc/VEN/A131/1988/G3P[7]	A131	Porcine	1988	VEN	G3	P[7]	I5	R1	C2	M1	A1	N1	T1	E1	H1
RVA/Pig-tc/VEN/A253/1988/G11P[7]	A253	Porcine	1988	VEN	G11	P[7]	I5	R1	C2	M1	A1	N1	T1	E1	H1
RVA/Pig-tc/ESP/OSU-C5111/2010/GP[7]	OSU-C511	Porcine	2010	ESP	G5	P[7]	I5	R1	C1	M1	A1	N1	T1	E1	H1
Human															
RVA/Human-tc/USA/Wa/1974/G1P[8]	Wa	Human	1974	USA	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-tc/USA/DS-1/1976/G2P[4]	DS-1	Human	1976	USA	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-tc/JPN/AU-1/1982/G3P[9]	AU-1	Human	1982	JPN	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
RVA/Human-tc/USA/HCR3A/1984/G3P[3]	HCR3A	Human	1984	USA	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Human-tc/ISR/Ro1845/1985/G3P[3]	Ro1845	Human	1985	ISR	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Human-wt/ITA/PAH136/1996/G3P[9]	PAH136	Human	1996	ITA	G3	P[9]	I2	R2	C2	M2	A3	N1	T6	E2	H3
RVA/Human-wt/ITA/PA158/1996/G3P[9]	PA158	Human	1996	ITA	G3	P[9]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Human-tc/ITA/PA260-97/1997/G3P[3]	PA260-97	Human	1997	ITA	G3	P[3]	I3	R3	C3	M3	A15	N2	T3	E3	H6
RVA/Human-tc/THA/T152/1998/G12P[9]	T152	Human	1998	THA	G12	P[9]	I3	R3	C3	M3	A12	N3	T3	E3	H6
RVA/Human-wt/THA/CMH222/2001/G3P[3]	CMH222	Human	2001	THA	G3	P[3]	I8	-	-	-	-	-	-	E3	-

Virus	Strain	Species	Year	Country	Gene										
					VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Human-wt/THA/CMH079/2005/G3P[10]	CMH079	Human	2005	THA	G3	P[10]	I8	-	-	-	-	-	-	E3	H6
RVA/Human-tc/CHN/L621/2006/G3P[9]	L621	Human	2006	CHN	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H6
RVA/Human-tc/THA/CU-365/2008/G3P[9]	CU365	Human	2008	THA	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H6
RVA/Human/BRA/R2638/2011/G3P[3]	R2638	Human	2011	BRA	G3	P[3]	-	-	-	-	-	-	-	-	-
RVA/Human-wt/BRA/1A3739/2011/G3P9	1A3739	Human	2011	BRA	G3	P[9]	I18	R3	C3	Mx	A19	N3	T3	E3	H6
RVA/Human-wt/CHN/E2451/2011/G3P[9]	E2451	Human	2011	CHN	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H6
RVA/Human-wt/JPN/12638/2014/G3P[3]	12638	Human	2014	JPN	G3	P[3]	I3	R3	C3	M3	A9	N2	T3	E3	H6
RVA/Human-wt/THA/MS2015-1-0001/2015/G3P[10]	MS2015-1-0001	Human	2015	THA	G3	P[10]	I8	R3	C3	M3	A9	N3	T3	E3	H6

4.3.6 Genetic diversity of RVA by reassortment analysis

The vistaplot and bootscan analysis were done to detect the reassortment events of RVAs in this study. The reassortment events were detected by the breakpoint (the point of which 2 sequences were exchanged) and high percentage of permuted trees (Y-Axis) from bootscan analysis.

In dogs, RV25012 was selected as the representative of dog's RVA in this study to use as query sequence to do the analysis. Three other closely related RVA strains were included in the analysis, including dog's RVA strain CU132 from previous report in Thailand (Charoenkul et al., 2020), human's RVA strain 12638 found in Japan (Okitsu et al., 2018), and cat's reference RVA strain FRV348 (Nakagomi et al., 2018).

The result from vistaplot analysis showed that dog's RVA strain CU132 (Charoenkul et al., 2020) had high similarity with dog's virus in this study (RV25012) along the concatenated sequence, except the NSP3 gene part. Moreover, human's RVA strain 12638 (Okitsu et al., 2018) had high similarity with RV25012 in VP6 gene and also in some part of VP7, VP1, VP2, NSP1, and NSP5 gene. While cat's reference RVA strain FRV348 (Nakagomi et al., 2018) had high similarity with RV25012 in some part of VP4, VP6, VP3 and NSP4 gene. It is noted that in NSP3 gene, FRV348 had the highest similarity with RV25012, among these 3 closely related strains. The vistaplot analysis of dog's RVA in this study (RV25012) showed in Figure 35. For bootscan analysis, the result showed that there was the possibility of reassortment events of dog's RVA in this study from these 3 closely related RVA strains. The analysis showed 6 obvious breakpoints between VP7-VP4, VP4-VP6, VP6-VP1, VP3-NSP1, NSP2-NSP3, and NSP4-NSP5. The RVA strains with high percentage of permuted trees may indicated the reassortment events from that strain in that segment of the virus. The result might be concluded that 4 gene segments which included VP4, VP2, VP3, NSP5 were reassorted from dog's RVA strain CU132, one gene segment from human's RVA strain 12638, which was VP6, and 2 gene segments might be from cat's reference RVA strain FRV348, which were NSP3 and NSP4. It is noted that in VP7, VP1, NSP1, and NSP2 gene, the bootscan analysis showed the inconclusive pattern which might result from the reassortment of other strains of RVA or recombinant events from

many RVA strains in this gene segments. The bootscan analysis of dog's RVA in this study (RV25012) showed in Figure 36.

In cats, RV25045 was use as query sequence to do the analysis. Four closely related RVA strains were selected to include in the analysis, including 2 bat's RVAs from China, strain MSLH14 (He et al., 2013), and strain MYAS33 (Xia et al., 2014), one from human's RVA strain MS2015-1-0001 found in Thailand (Komoto et al., 2021), and one simian's RVA strain TUCH reported in USA (Brown et al., 2011).

The result from vistaplot analysis showed that none of these 4 RVAs strains had high similarity with cat's RVA in this study (RV25045) along the concatenated sequence. However, bat's RVA strain MYAS33 (Xia et al., 2014) had high similarity with cat's RVA in this study in VP6, and some part of NSP3 and NSP4 gene. Moreover, human's RVA strain MS2015-1-0001 (Komoto et al., 2021) also had high similarity with RV25045 in some part of VP3, NSP4, and NSP5 gene. While virus from simian strain TUCH (Brown et al., 2011) had high similarity with RV25045 in some part of VP2 gene. It is also noted that bat's RVA strain MSLH14 (He et al., 2013) had the highest similarity with RV25045 in VP4 gene, among these 4 closely related strains. The vistaplot analysis of cat's RVA in this study (RV25045) showed in Figure 37. For bootscan analysis, the result showed that there was the possibility of reassortment events of cat's RVA in this study from these 4 closely related RVA strains. The analysis showed 8 obvious breakpoints between VP7-VP4, VP4-VP6, VP1-VP2, VP2-VP3, VP3-NSP1, NSP1-NSP2, NSP2-NSP3, and NSP3-NSP4. The RVA strains with high percentage of permuted trees may indicated the reassortment events from that strain in that segment of the virus. The result might be concluded that VP4 was the reassortment of bat's RVA strain MSLH14, 2 gene were reassorted from another bat's virus strain MYAS33, which were VP6 and NSP3 gene. While VP3 and NSP4 gene were reassorted from human's virus strain MS2015-1-0001, the last was VP2 gene, which was the reassortment of simian's virus strain TUCH (Brown et al., 2011). It is noted that in VP7, VP1, NSP1, NSP2, and NSP5 gene, the bootscan analysis showed the inconclusive pattern which might result from the reassortment of other strains of RVA or recombinant events from many RVA strains in this gene segments.

The bootscan analysis of cat's RVA in this study (RV25045) showed in Figure 38.

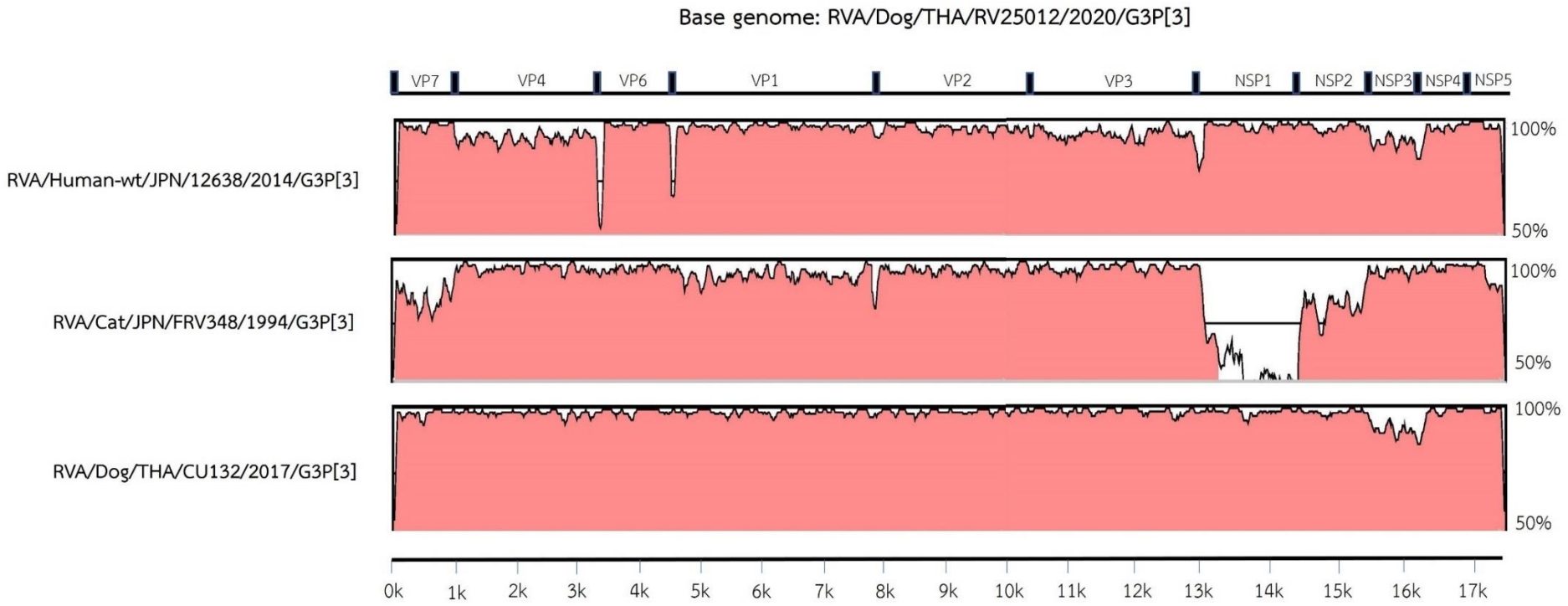


Figure 35: Vistaplot analysis between dog's RVA in this study (RV25012) and closely related strains

X-axis; Nucleotide position, Y-axis; Percentage of similarity.

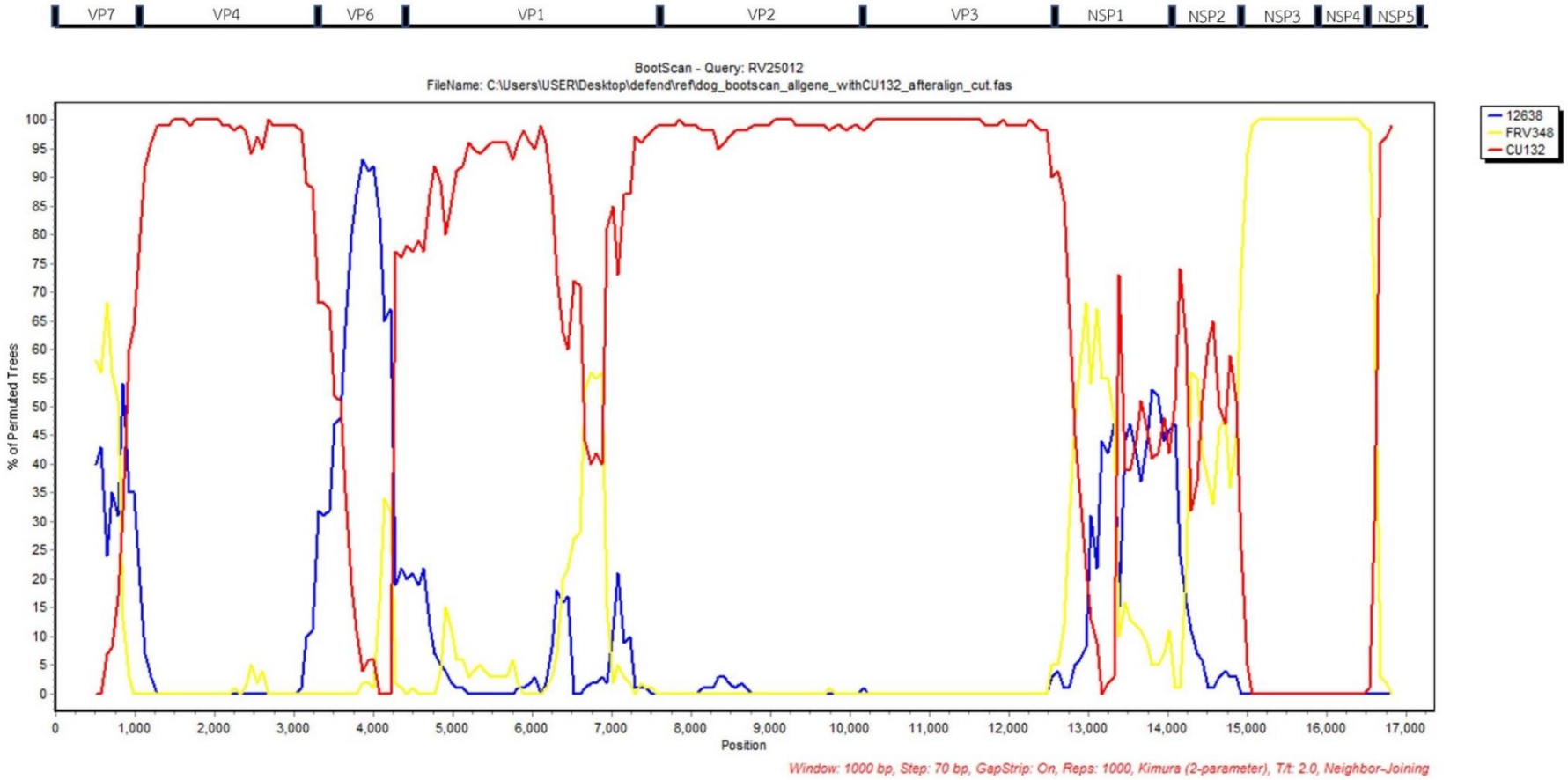


Figure 36: Bootscan analysis between dog's RVA in this study (RV25012) and closely related strains

X-axis; Nucleotide position, Y-axis; Percentage of permuted trees, Red line; strain CU132, Blue line; strain 12638, Yellow line; strain FRV348.

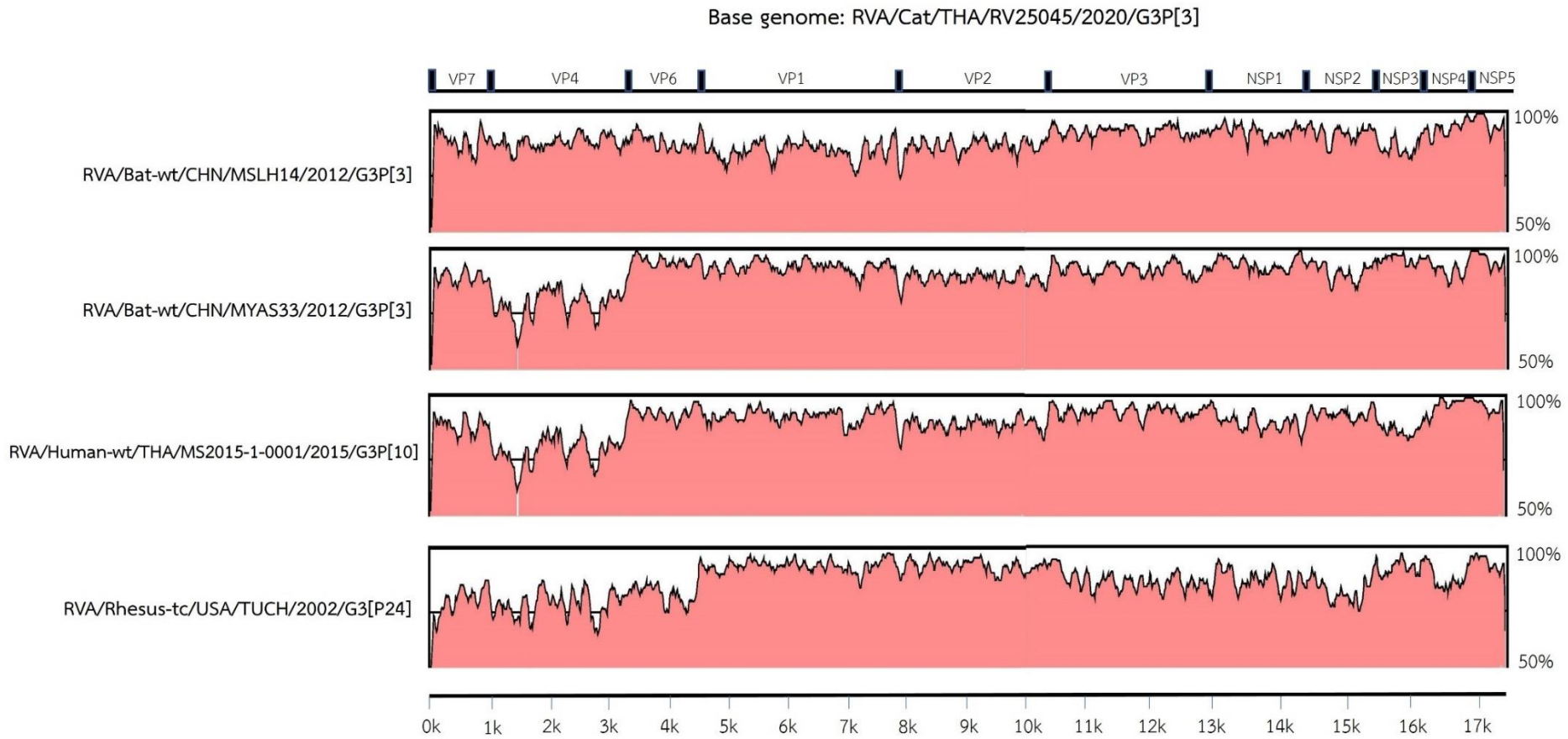


Figure 37: Vistaplot analysis between cat's RVA in this study (RV25045) and closely related strains

X-axis; Nucleotide position, Y-axis; Percentage of similarity.

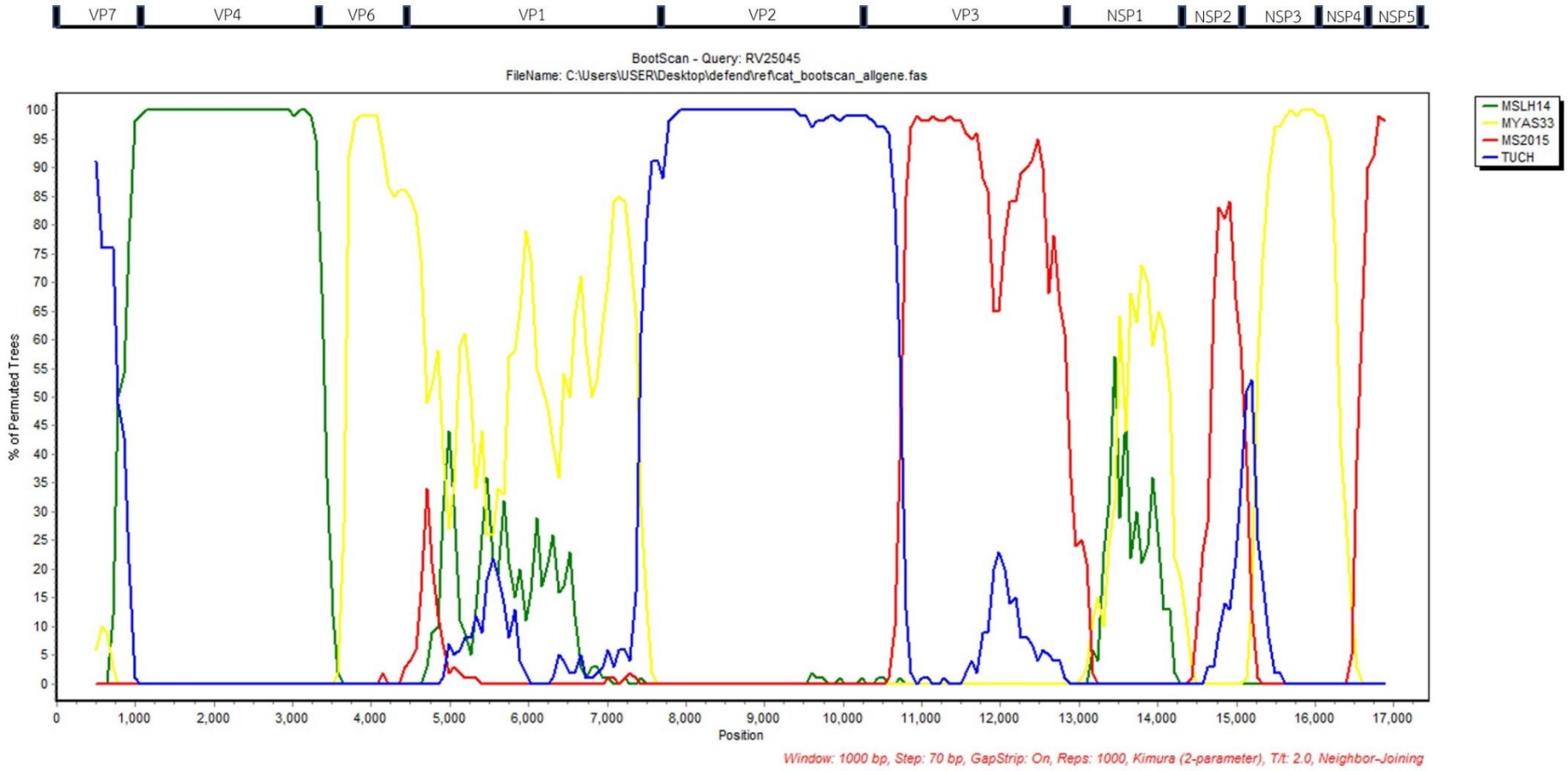


Figure 38: Bootscan analysis between cat's RVA in this study (RV25045) and closely related strains

X-axis; Nucleotide position, Y-axis; Percentage of permuted trees, Red line; strain MS2015-1-0001, Blue line; strain TUCH, Yellow line; strain MYAS33, Green line; strain MSLH14.

Chapter 5
Discussion

5.1 Occurrence of RVA in dogs and cats in Bangkok and vicinity.

In this study, from 572 rectal swab samples from dogs and cats, the occurrence of rotavirus A (RVA) was 1.92% (11/572). By species, we found 2.75% (8/290) occurrence of canine rotavirus A (CRVA) in dogs which was higher than the occurrence in the previous study in Thailand which was only 0.7% (5/710) (Charoenkul et al., 2020). However, the occurrence of CRVA in dogs in this study was lower than those in other studies in other countries, which were 39.7% (31/78) in Germany (Otto et al., 2015), 16.33% (16/98) in Iran (Mosallanejad et al., 2015), 40% (18/45) in Mexico (Ortega et al., 2017) and 8.2% (15/184) in Brazil (Alves et al., 2018). In cats, we found only 1.06% (3/282) occurrence of feline rotavirus A (FRVA), which was lower than the occurrence in other studies, which were 50% (23/46) in Germany (Otto et al., 2015) and 3% (57/1727) in United Kingdom (German et al., 2015). To the best of our knowledge, this thesis is the first to report FRVA in Thailand.

By age of animals, according to life stage of dogs and cats, we found 4.88% (6/123) occurrence of rotavirus A in puppy and kitten. In dogs, we found occurrence of CRVA in only two life stage, which were 7.81% (5/64) in puppy life stage, and 6.12% (3/49) in senior life stage. While in cats, there were 1.69% (1/59) and 2.41% (2/83) occurrence of FRVA in kitten and adult life stage, respectively. In detail, there were 54.55% (6/11) of animals age less than 6 months (puppy/kitten life stage). Moreover, 62.5% (5/8) of dogs with positive CRVA were ages less than 6 months (puppy life stage), which were the same as other studies that most positive animal were the young age animals (Mosallanejad et al., 2015; Charoenkul et al., 2020). This result speculated that age is one of the risk factors affecting the occurrence of RVA in dogs but should be confirm by statistical analysis, while in cats the result is still inconclusive.

5.2 Genetic diversity of RVA in dogs and cats

5.2.1 Genotype of RVA in dogs and cats

For genotyping of RVA in this study, 2 viruses from dogs (RV25012, DC25170) were identified as genotype G3P[3], which are the same RVA genotype as previous study in Thailand (Charoenkul et al., 2020). Our result supported the additional evidence that G3P[3] is the predominant genotype circulating in dogs in Thailand which similar to RVA genotype in dogs worldwide (Tsugawa and Hoshino, 2008; Martella et al., 2010; Matthijnssens et al., 2011b; Doro et al., 2015; Mihalov-Kovács et al., 2015). It is noted that, the G3P[3] genotype still the only genotype ever reported in dogs. For genotyping of RVA in cats, the virus (RV25045) was classified as genotype G3P[3], which is the first report of feline rotavirus A (FRVA) in Thailand. Notably, the FRVA genotype G3P[3] had been reported in cats worldwide (Martella et al., 2011; German et al., 2015; Nakagomi et al., 2018). Apart from FRVA genotype G3P[3] in cats, there were other genotypes ever reported in cats e.g. G3P[9] (Martella et al., 2011; Nakagomi et al., 2018), and G6P[9] (German et al., 2015). The RVA genotype G3P[3] also had been found in other animal species such as strain E3198 in horse (Mino et al., 2013), strain LUS12-14, and MSLH14 in bats (He et al., 2013; Sasaki et al., 2016). It is interesting to noted that the genotype G3P[3] had also been reported in human in several countries such as strain HCR3A in USA, strain Ro1845 in Israel (Tsugawa and Hoshino, 2008), strain PA260-97 in Italy (De Grazia et al., 2007), strain R2638 in Brazil (Luchs et al., 2012), strain 12638 in Japan (Okitsu et al., 2018) and strain CMH222 in Thailand (Khamrin et al., 2006). These publications supports the speculation of interspecies transmission and zoonotic potential of RVA genotype G3P[3].

For the RVA classification based on all 11 genes (VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5), our results showed that 2 RVAs from dogs (RV25012, DC25170) were classified into genotype G3-P[3]-I3-R3-C3-M3-A9-N2-T3-E3-H6 which are the same genotype as previous report in Thailand (Charoenkul et al., 2020). It is interesting to note that genotype C3 of VP2 gene found in dogs in Thailand had never been reported in dogs in other countries, but had been reported in other species such as cat (Nakagomi et al., 2018), horse (Mino et al., 2013), bat (He et al.,

2013) , and human (Okitsu et al., 2018), which might be the result from reassortment of the virus in these animal species.

On the other hand, the virus from cat (RV25045) was classified into RVA genotype G3-P[3]-I8-R3-C3-M3-A9-N3-T3-E3-H6. According to the report worldwide, the genotype I8 of VP6 gene has never been reported in cats before, but had been found in bats in China, strain MSLH14 (He et al., 2013), and strain MYAS33 (Xia et al., 2014). Interestingly, genotype I8 of VP6 gene had also been reported in 3 human's virus in Thailand which include strain CMH222 (Khamrin et al., 2006), strain CMH079 (Khamrin et al., 2009), and strain MS2015-1-0001 (Komoto et al., 2021). Our result suggested that RVAs had been circulating in cats, bats, and human, and the reassortment event of RVAs in these animals and/or human might have been occurred.

5.2.2 Genetic constellation of RVA in dogs and cats

The combination of 11 genotypes from each gene segment were analyzed and called as genetic constellation. In this study 2 viruses from dogs in this study (RV25012, DC25170) had the G3-P[3]-I3-R3-C3-M3-A9-N2-T3-E3-H6 genotype, which belongs to AU-1 genetic constellation. The dog's RVAs genetic constellation have composition of gene segments of both AU-1-like (VP2) and Cat-like genogroup (10 segments), same as previous study in Thailand (Charoenkul et al., 2020). From our knowledge, these genetic constellations have never been reported in dogs in other countries but one study from Japan reported this genetic constellation in human's virus strain 12638 (Okitsu et al., 2018), which could support the evidence of zoonotic potential of this RVA in dogs.

RVA from cat in this study (RV25045) was classified as genotype G3-P[3]-I8-R3-C3-M3-A9-N3-T3-E3-H6 genotype, which also belongs to AU-1 genetic constellation. This cat's RVA genetic constellation has composition of gene segments of AU-1-like (VP2), Cat-like genogroup (9 segments), and one additional unique genotype of VP6 gene segment (I8), which makes this genetic constellation unique from other RVAs in cats. Interestingly, this genetic constellation had been reported once in China's bat

strain MSLH14 (He et al., 2013), which could support the evidence of interspecies transmission between RVAs in cat and bat species.

5.2.3 Interspecies transmission, reassortment event, and zoonotic potential of RVA in dogs and cats

Based on the result from phylogenetic analysis, nucleotide identities, and bootscan analysis, dog's RVAs in this study (RV25012, DC25170) could have been the result of multiple reassortment from dog, human, and cat's viruses. Our result found that 2 dog's RVAs in this study (RV25012, DC25170) had closest genetic relationship with dog's RVAs from previous report in Thailand (Charoenkul et al., 2020) in 9 segments (VP1, VP2, VP3, VP4, VP7, NSP1, NSP2, NSP4, NSP5) with around 98-99% nucleotide and amino acid identities, but in VP6 gene, it was closest to human's virus strain 12638 found in Japan (Okitsu et al., 2018) with 99% nucleotide and amino acid identity. The result of bootscan analysis also showed that VP6 gene might be the result of reassortment from this human strain. Interestingly, this human's virus also had same genetic constellation with dog's virus in this study, which could support the evidence of zoonotic potential of dog's RVAs in this study. Moreover, NSP3 gene of dog's virus in this study had closest genetic relationship with cat's reference RVA strain FRV348 (Nakagomi et al., 2018) with 95-98% nucleotide and amino acid identity and bootscan analysis also showed the result of reassortment event of NSP3 and NSP4 gene from this cat strain. The schematic presentation of possible multiple reassortment of dog's RVAs in this study is shown in Figure 39. The multiple reassortment of RVA in dogs had also been reported in other studies (Matthijnssens et al., 2011b) including the previous study in Thailand which reported the reassortment of RVA originated from human and bats (Charoenkul et al., 2020). Moreover, there were also many reports of canine-like RVA found in human (De Grazia et al., 2007; Grant et al., 2011; Luchs et al., 2012; Okitsu et al., 2018), and these canine-like human RVA were all classified in genotype G3P[3], same as all dog RVAs. It should be noted that the inconclusive result of bootscan analysis in VP7, VP1, NSP1, and NSP2 gene might be the result of reassortment from other strains of the virus, or might be the intragenic recombination of the virus from many strains,

which might be rare but had been reported in some studies in human (Parra et al., 2004; Phan et al., 2007; Esona et al., 2017; Hoxie and Dennehy, 2020) but never been reported in dog. Thus, this study could support the evidence of reassortment event, and the possibility of intragenic recombinant occurred in dog's RVA and the zoonotic potential of the virus.

For RVA in cat in this study (RV25045), the result from phylogenetic analysis, nucleotide identities, and bootscan analysis showed that RV25045 had closest genetic relationship with human's virus found in Thailand, which include strain CMH079 in VP7 gene, and strain CMH222 (Khamrin et al., 2006) in VP4 gene, as well as strain MS2015-1-0001 (Komoto et al., 2021) in VP3, NSP2, NSP4, NSP5 genes, with around 87-99% nucleotide and amino acid identities. The bootscan analysis also showed that the reassortment event from human's virus strain MS2015-1-0001 might occurred in VP3 and NSP4 gene. This could support the evidence of zoonotic potential of this virus strain. It should be noted that human's virus strain CMH222 and CMH079 were not included in bootscan analysis due to incomplete whole genome sequences of both strains, which unfortunately because it could be helpful for the analysis of reassortment or interspecies transmission and probable zoonotic potential of the virus. In other segments, there were bat's RVA found in China that had closest genetic relationship with cat's RVA in this study, which were strain MSLH14 (He et al., 2013) in VP4 gene, and strain MYAS33 (Xia et al., 2014) in VP6, NSP1, NSP3, and NSP5 gene, with around 87-100% nucleotide and amino acid identities. In VP1 and VP2 genes, cat's RVA in this study had closest genetic relationship with simian's RVA strain TUCH reported in USA (Brown et al., 2011), with 93-98% nucleotide and amino acid identities. The bootscan analysis had showed the result of reassortment from bat's virus strain MSLH14 in VP4 gene, and from strain MYAS33 in VP6 and NSP3 gene, and from simian in VP2 gene. It should be noted that in VP4 gene, even though the closest genetic relationship with cat's virus in this study were human's virus strain CMH222 and bat's virus strain MSLH14, but the nucleotide and amino acid identities were low compared to other segment, with only 87% (nt) and 95-97% (aa), this result might suggest that there might be the reassortment of this segment from other RVAs that never had been discovered.

Interestingly, even though cat's RVA in this study had the same genotype with some cat's reference strain in almost every segment (except VP6), but there were none of any segments were closely related to cat's reference strain by phylogenetic analysis or nucleotide/amino acid identities. Moreover, cat's RVA in this study was more likely to related to bat-like RVA strains and had the same genetic constellation with bat's virus strain MSLH14 (He et al., 2013). The human's RVA strain MS2015-1-0001 found in Thailand which was closely related with cat's RVA in this study were also closely related to bat or bat-like RVA and had showed the evidence of direct interspecies transmission from bat to human (Komoto et al., 2021), while human's RVAs strain CMH222 and strain CMH079 together with simian's RVA strain TUCH had showed the possibility of multiple reassortment from many animal species (Khamrin et al., 2006; Khamrin et al., 2009; Matthijnssens et al., 2010). Multiple reassortment of RVA in cats had also been reported in some studies (Martella et al., 2011; Matthijnssens et al., 2011b). Thus, the origin of cat's RVA in this study is still inconclusive, it might speculate that direct interspecies transmission of the virus from other intermediate species to cats might occur and the reassortment event might happen in those intermediate species. The schematic presentation of possible direct interspecies transmission of cat's RVA in this study is shown in Figure 40. Moreover, bat and simian's RVAs that closely related with cat's RVA in this study were from other countries. Since the study of RVA in these species in Thailand are still limited, so further study of RVA in bats, simians, and other wildlife species in Thailand will be helpful for the analysis and understanding the origin of cat's RVA and genetic relationship among these species.

In conclusion, this study had found the occurrence of RVAs in dogs and cats in Thailand, which was lower compared to the studies in other countries. We also found that age could be the risk factor affecting the occurrence of RVAs in dogs, but the result in cats is still inconclusive. In dog's RVAs, we had found the evidence of multiple reassortment from dog, human, and cat's RVAs. While in cat RVA, we found that the virus might had the direct interspecies transmission from intermediate species to cat and the reassortment event might occurred in that intermediate species. The zoonotic potential had also been speculated in both dog and cat RVAs.

Furthermore, the study of RVAs in bat, simian and other wildlife species in Thailand should be done to find the origin of cat's RVA and to understand the genetic relationship among these species and also the genetic evolution of the virus.

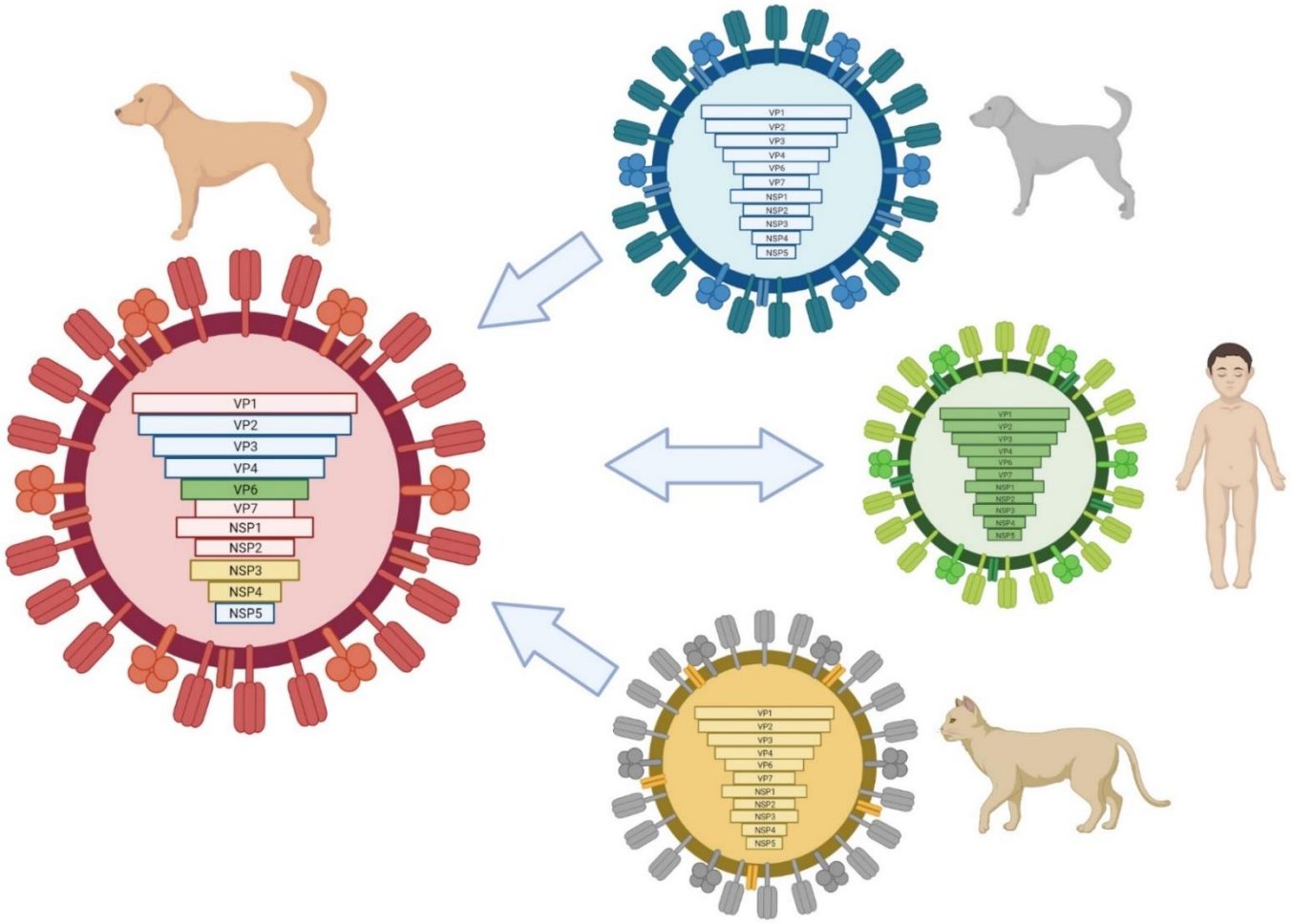


Figure 39: Schematic presentation of possible multiple reassortment of dog's RVA in this study

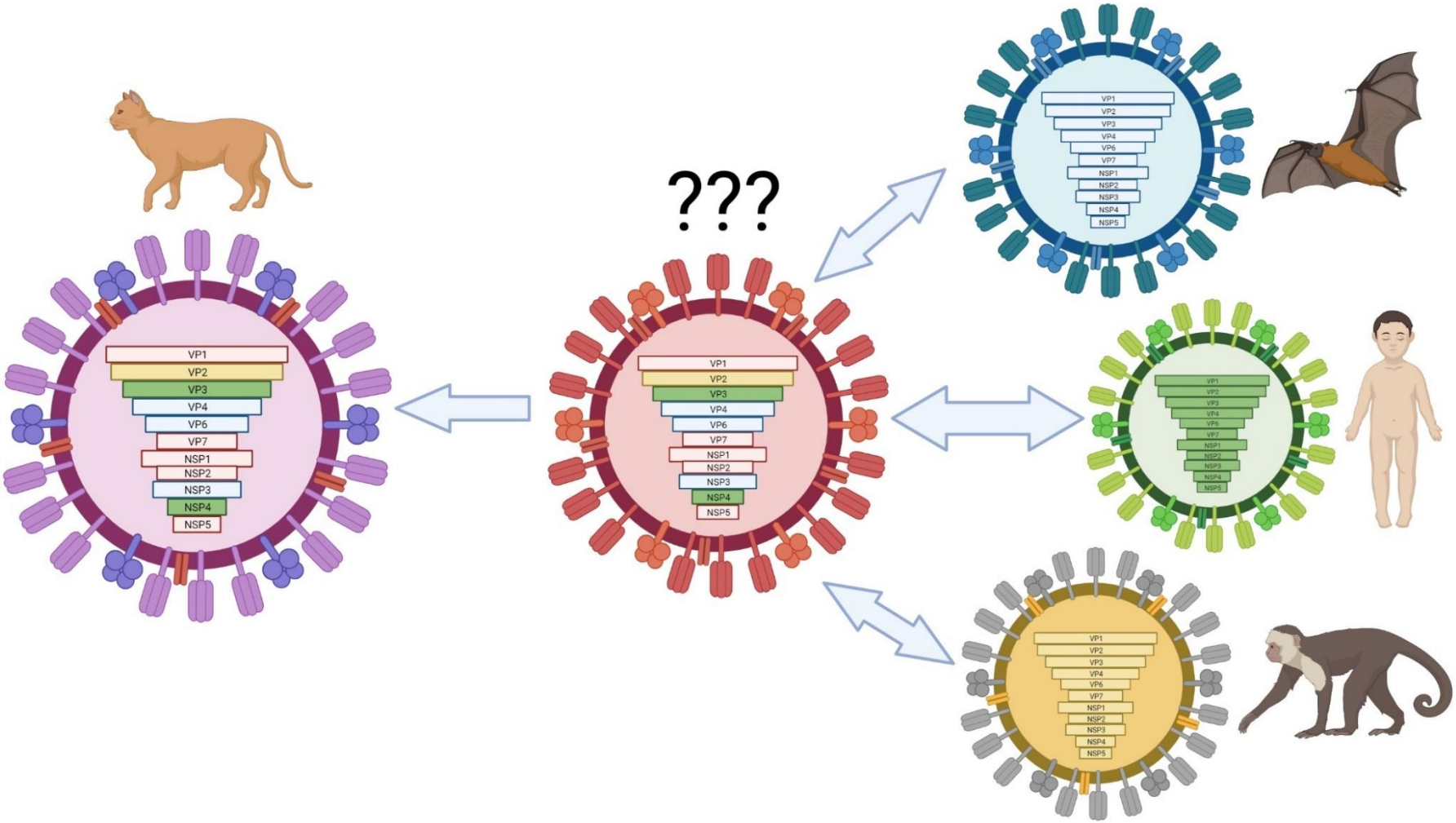


Figure 40: Schematic presentation of possible interspecies transmission of cat's RVA in this study

Conclusion

Rotavirus A (RVA) in one of the important pathogens causing gastroenteritis in human and many animal species. The zoonotic potential of RVA has been reported and raises major concerns, especially in high animal-human interface settings. The objectives of this thesis were to determine the occurrence of RVA in dogs and cats, and to investigate the genetic diversity and genetic relationship among the RVAs from dogs, cats, human, and other animal species. The total of 572 rectal swab samples were collected from dogs and cats in animal hospitals, in Bangkok and vicinities, during January 2020 to June 2021 (18 months). Our result found 1.92% (11/572) occurrence of RVA in dogs and cats, which by species were 2.75% (8/290) in dogs, and 1.06% (3/282) in cats. Age of animals might be the risk factor affecting the occurrence of RVAs in dogs and should be confirm with statistical analysis, but the result in cats is still inconclusive. Besides, our finding is the first report of RVA in cats in Thailand.

Two RVAs from dogs (RV25012, DC25170) and one RVA from cat (RV25045) were subjected to whole genome sequencing. The genetic characterization of the virus showed that all 3 RVAs were classified as RVA genotype G3P[3]. In detail, genetic constellation of dog's RVAs had the G3-P[3]-I3-R3-C3-M3-A9-N2-T3-E3-H6 genotype, which is the same as that in the previous study in Thailand but different genetic constellation from dog's RVAs in other countries. Moreover, this genetic constellation had been reported in human's RVA, suggesting the zoonotic potential of this virus strain. While genetic constellation of RVA from a cat had G3-P[3]-I8-R3-C3-M3-A9-N3-T3-E3-H6 genotype, which never been reported in cats before, but was found in bat in China, suggesting the possible of interspecies transmission of this virus strain. Both dog and cat's RVAs genetic constellation have composition of gene segments of AU-1-like and Cat-like genogroup.

The phylogenetic analysis and pairwise comparison analysis showed that dog's RVAs in this study were closely related and had high nucleotide/amino acid identities to dog's RVAs from previous reported in Thailand in 9 segment (98-99% nt

and aa), except VP6 gene which were closely related to human's RVA in Japan (99% nt and aa), and NSP3 gene which was closely related to cat's reference strain (95-98% nt and aa). The result of bootscan analysis also supported the possible reassortment of these dog's RVAs, suggesting the possible multiple reassortments from dog, human, and cat's viruses. On the other hands, RVA from cat in this study (RV25045) was closely related to RVA in human, bat, and simian in different segments (87-100% nt and aa), suggesting possible of multiple reassortment and zoonotic potential of this virus. Moreover, the result of bootscan analysis also showed the possible reassortment in this RVA. However, it should be noted that none of any segments of cat's RVA in this study were closely related to cat's reference strain, which might indicate that interspecies transmission of the RVA from some animal species to cat had been occurred, and the reassortment event might have happened before in those animal species. Thus, this study had reported the occurrence of RVA in dogs and cats, and suggesting the possible multiple reassortment, interspecies transmission, as well as zoonotic potential of the viruses.

Based on the result of this thesis, it has raised public health awareness on the zoonotic potential of RVAs in dogs and cats. Thus, the following recommendations should be provided

- The households with dogs or cats (especially puppy) should have personal hygiene practice, such as "frequent handwashing" especially after playing with pets, to protect the zoonotic diseases cause by RVAs from dogs or cats, especially in children.
- The household areas and pet cages should be regularly cleaned and sanitized.
- The close contact behaviors with dogs and cats such as "human face licking" should be avoid.
- The detection of RVAs infection in dogs and cats (especially puppy) should be considered by veterinarians due to its public health concern.

- The risk communication with high-risk occupations such as veterinarians, vet-nurses, and shelter/kennel's caretakers should also be provided. The personal protective equipment (PPE) including gloves, surgical mask, and protective suit (e.g. gown, scrub suit, boots, etc.) should be used when handling with animals or when working in the facilities with crowded animals. The personal hygiene should also be reminded.
- Routine cleaning of the facilities with crowded animals such as animal hospitals, animal shelters, dog/cat hotels, and dog/cat kennels should be done.

These practices will help minimize risk of RVAs transmission and infection as well as reduce a chance of reassortment event, interspecies transmission, and evolution of RVAs.

Recommendations for further studies are 1) study of RVAs in larger scale in dogs and cats to determine the occurrence, distribution, and genetic diversity of RVAs should be performed in other provinces in Thailand, and/or in other facilities with crowded animals such as animal shelters, animal kennels, etc. 2) study of RVAs in bats, simians, and other wildlife species in Thailand should be done for better understand the origin and evolution of the virus, and to determine the genetic relationship among the RVAs in the future.

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VITA

NAME	Ekkapat Chamsai
DATE OF BIRTH	22 Feb 1991
PLACE OF BIRTH	Bangkok, Thailand