

TARGETED SURVEILLANCE OF POTENTIAL ZONOTIC RESPIRATORY AND ENTERIC  
VIRUSES IN DOGS IN BANGKOK, THAILAND



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การเฝ้าระวังเชื้อไวรัสก่อโรคสัตว์สู่มนุษย์ ในระบบทางเดินหายใจ และทางเดินอาหารในสุนัขแบบมุ่งเป้า ในเขตกรุงเทพมหานคร ประเทศไทย



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต  
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กมลพรรณ เจริญกุล : การเฝ้าระวังเชื้อไวรัสก่อโรคสัตว์สู่คน ในระบบทางเดินหายใจ และทางเดินอาหารในสุนัขแบบมุ่งเป้า ในเขตกรุงเทพมหานคร ประเทศไทย. ( TARGETED SURVEILLANCE OF POTENTIAL ZONOTIC RESPIRATORY AND ENTERIC VIRUSES IN DOGS IN BANGKOK, THAILAND ) อ.ที่ปรึกษาหลัก : ศ. น.สพ.ดร.อลงกร อมรศิลป์D.V.M., Ph.D.

สุนัขเป็นสัตว์ที่มีความใกล้ชิดกับคน ทำให้มีความเสี่ยงในการติดต่อหรือแพร่กระจายเชื้อไวรัสก่อโรคสัตว์สู่คนได้มากขึ้น วิทยานิพนธ์ฉบับนี้ มีวัตถุประสงค์เพื่อสำรวจและถอดรหัสพันธุกรรมเชื้อไวรัสก่อโรคสัตว์สู่คนในสุนัขและคน โดยเฉพาะอาชีพที่มีความใกล้ชิดกับสุนัข วิทยานิพนธ์ฉบับนี้ประกอบด้วย 8 หัวข้อ โดยผลการศึกษาในหัวข้อที่หนึ่งถึงสาม เป็นการสำรวจเชื้อไวรัสในระบบทางเดินหายใจของสุนัข ได้แก่ parainfluenzavirus type 5 (PIV-5) canine influenza virus (CIV) และ coronavirus (CoV) โดยเก็บตัวอย่างปัสสาวะสุนัขที่แสดงอาการระบบทางเดินหายใจ ตั้งแต่ พฤศจิกายน 2558 ถึง ธันวาคม 2561 ผลการศึกษาพบอุบัติการณ์ของเชื้อ PIV-5 CIV และ CoV คิดเป็น 5.6% (32 /571), 1.4% (8/571) และ 13.1% (75/571) ตามลำดับ และผลการถอดรหัสพันธุกรรมพบว่าเชื้อ PIV-5 ในไทยมีความใกล้เคียงกับเชื้อไวรัสในประเทศจีนและเกาหลี แต่อย่างไรก็ตามเชื้อไวรัสที่พบในสุนัขต่างจาก PIV-5 ที่รายงานในคน ส่วนผลการศึกษารหัสพันธุกรรมของเชื้อ CIV พบว่าเชื้อไวรัสที่ได้เป็น pandemic H1N1/2009 ซึ่งเหมือนกับไวรัสไข้หวัดใหญ่ที่พบในคนและในสุกรในประเทศไทย ผลงานวิจัยนี้ชี้ให้เห็นว่า สุนัขสามารถติดเชื้อไวรัสไข้หวัดใหญ่ชนิด pandemic H1N1/2009 ที่อาจจะมีส่วนกำเนิดมาจากคนได้ ส่วนผลการศึกษารหัสพันธุกรรมของเชื้อ CoV พบว่า เชื้อ CoV ในระบบทางเดินหายใจในสุนัข อยู่ในกลุ่มเบต้าโคโรนา ซึ่งมีความใกล้เคียงกับเชื้อไวรัสที่พบในคน (HCoV-OC43) และวัว (BCoV) ซึ่งต่างจากเชื้อไวรัส CoV ที่ก่อให้เกิดโรคในระบบทางเดินอาหารในสุนัข ในกลุ่มแอลฟาโคโรนา ผลการวิเคราะห์หาช่วงเวลาที่เป็นจุดกำเนิดร่วมของไวรัส พบว่าเชื้อไวรัส CoV ในสุนัขนั้น อาจพัฒนาจากเชื้อไวรัส CoV ในคน (HCoV-OC43) และวัว (BCoV) ตั้งแต่ 2547 การศึกษาในหัวข้อที่สี่ถึงเจ็ด เป็นการสำรวจไวรัสในระบบทางเดินอาหารในสุนัข ได้แก่ Kobuvirus (KoV) Norovirus (NoV) Rotavirus (RoV) และ Canine parvovirus type 2 (CPV-2) ซึ่งเชื้อไวรัสเหล่านี้สามารถก่อโรคในสุนัข และเชื้อไวรัสบางชนิดมีรายงานการติดต่อจากสุนัขสู่คนได้ โดยเก็บตัวอย่างปัสสาวะสุนัขที่แสดงอาการระบบทางเดินอาหาร ผลการศึกษาพบอุบัติการณ์ของเชื้อ KoV NoV CPV-2 และ RoV ในสุนัข คิดเป็น 17.6% (54/307), 11.1% (2/18), 29.9% (133/444) และ 0.7% (5/710) ตามลำดับ ผลการถอดรหัสพันธุกรรมของเชื้อ Kobuvirus พบว่าเชื้อไวรัสในประเทศไทย มีความใกล้เคียงกับเชื้อไวรัส KoV ในสุนัขในประเทศจีน ส่วนผลการถอดรหัสพันธุกรรมของเชื้อไวรัส NoV ในสุนัข พบว่าเชื้อไวรัส NoV เป็นสายพันธุ์ GII.Pe-GII.4 Sydney ซึ่งมีความใกล้เคียงกับเชื้อ NoV ที่ทำให้เกิดการระบาดในคนในประเทศไทย นอกจากนี้พบว่าสุนัขมีประวัติใกล้ชิดกับคนที่ได้รับการยืนยันติดเชื้อ NoV มาก่อน ซึ่งผลการศึกษานี้ พบว่าสุนัขอาจจะได้รับเชื้อ NoV จากคนได้ ส่วนผลการถอดรหัสพันธุกรรมของเชื้อ CPV-2 รายงานนี้เป็นการตรวจพบเชื้อไวรัส CPV-2c เป็นครั้งแรกในไทย โดยเชื้อไวรัส CPV-2 มีพันธุกรรมใกล้เคียงกับในเอเชียมากกว่าในอเมริกาและยุโรป นอกจากนี้ยังพบว่าแมวสามารถติดเชื้อ CPV-2c และทำให้เกิดโรคในระบบทางเดินอาหารในแมวได้ด้วย ส่วนผลการถอดรหัสพันธุกรรมของเชื้อ RoV พบเชื้อ RoV ชนิด G3P[3] มากที่สุด และรหัสพันธุกรรมทั้งตัวของไวรัส มีรูปแบบ G3-P[3]-I3-R3-C3-M3-A9-N2-T3-E3-H6 ซึ่งเป็นครั้งแรกที่มีการตรวจพบเชื้อ RoV รูปแบบดังกล่าวในสุนัข นอกจากนี้ผลการวิเคราะห์หาช่วงเวลาที่เป็นต้นกำเนิดร่วมของเชื้อไวรัส พบว่า RoV อาจมีการแลกเปลี่ยนยีนกับเชื้อไวรัสจาก คน ค้างคาว และสุนัขก่อนมีการพัฒนาของไวรัส G3P[3] ในสุนัขในประเทศไทย ผลการศึกษาในข้อที่ 8 ได้แก่การสำรวจเชื้อไวรัสที่ก่อให้เกิดโรคสัตว์สู่คนในคน โดยเก็บตัวอย่างปัสสาวะ อุจจาระ และการตอบสนองแบบสอบถาม ในบุคคลที่มีอาชีพกลุ่มเสี่ยงที่ใกล้ชิดกับสุนัขจำนวน 100 คน โดยตรวจหาเชื้อไวรัส Parainfluenzavirus (PIV) Influenzavirus (IVA) Coronavirus (CoV) Rotavirus (RoV) Norovirus (NoV) ผลการศึกษา พบเชื้อไวรัส CoV จากตัวอย่างปัสสาวะ จำนวน 2 ตัวอย่าง และตรวจไม่พบเชื้อไวรัสชนิดอื่นๆ ผลการถอดรหัสพันธุกรรมของเชื้อไวรัส CoV พบว่า เชื้อไวรัสมีความใกล้เคียงกับเชื้อไวรัส CoV สายพันธุ์ HCoV- 229E ซึ่งเคยมีรายงานมาก่อนในประเทศไทย ส่วนผลการศึกษาแบบสอบถาม พบว่า 52% ของผู้เข้าร่วมงานวิจัยระบุว่าคุณเองมีความเสี่ยงในการติดเชื้อโรคติดต่อจากสุนัขและ 8% ใส่ถุงมือทุกครั้งขณะปฏิบัติงานเมื่อต้องสัมผัสกับสุนัข เนื่องจากการศึกษาในครั้งนี้จำนวนตัวอย่างมีค่อนข้างน้อยและเป็นการเก็บตัวอย่างเพียงครั้งเดียว ดังนั้นควรมีการศึกษาในกลุ่มประชากรที่มากขึ้น โดยสรุปผลงานการศึกษาของวิทยานิพนธ์นี้พบว่า มีการปรากฏของเชื้อไวรัสในระบบทางเดินหายใจและทางเดินอาหารในสุนัขในประเทศไทย ซึ่งอาจจะก่อให้เกิดโรคติดต่อระหว่างสัตว์สู่คน และจากการศึกษาทางพันธุกรรมของเชื้อไวรัส ชี้ให้เห็นว่าเชื้อไวรัสมีวิวัฒนาการที่รวดเร็วและเปลี่ยนแปลงตลอดเวลา โดยเฉพาะเมื่อมีสายพันธุ์ใหม่หรือมีการติดเชื้อข้ามสายพันธุ์มายังสัตว์ ซึ่งผลการศึกษาในครั้งนี้เป็นประโยชน์ต่อบุคคลที่มีอาชีพกลุ่มเสี่ยงที่ใกล้ชิดกับสัตว์เลี้ยง เช่น สัตว์แพทย์ ผู้ช่วยสัตวแพทย์ เจ้าของสัตว์ ทำให้มีความรู้และความเข้าใจ เกี่ยวกับโรคติดต่อในระบบทางเดินหายใจและระบบทางเดินอาหารในสุนัขมากขึ้นและสามารถวางแผนการเฝ้าระวัง และควบคุมป้องกัน เชื้อไวรัสที่เป็นเชื้อไวรัสอุบัติใหม่และอุบัติซ้ำ และอาจทำให้เกิดโรคทั้งในคนและสัตว์

สาขาวิชา สัตวแพทยศาสตรบัณฑิต

ปีการศึกษา 2563

ลายมือชื่อนิสิต .....

ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Kamonpan Charoenkul : TARGETED SURVEILLANCE OF POTENTIAL ZOOBOTIC RESPIRATORY AND ENTERIC VIRUSES IN DOGS IN BANGKOK, THAILAND . Advisor: Prof. Dr. ALONGKORN AMONSIN, D.V.M., Ph.D.

Human-dog interface poses a risk of transmission and spread of zoonotic viruses. The objective of the thesis entitled is to survey and genetic characterize zoonotic viruses in dogs and humans who have high-risk occupations and in close contact with dogs. This thesis contains 8 topics. The results of the first to third topics were the surveillance of respiratory viruses in dogs including Canine Parainfluenza type 5 (CPiV-5), Canine influenza virus (CIV) and Coronavirus (CoV). The nasal swab samples were collected from dogs with respiratory signs during 2015-2018. The results showed that the occurrence of CPiV-5, CIV and CoV were 5.6% (32 /571), 1.4% (8/571) and 13.1% (75/571), respectively. The genetic analysis of CPiV-5 showed that Thai CPiV-5 were closely related with CPiV-5 from China and South Korea but they were different from Human PIV-5. The genetic analysis of CIV showed that Thai CIV subtype was pandemic H1N1/2009. The Thai CIV was closely related to pandemic H1N1/2009 infected in swine and human. This result suggested that dogs can be infected with pandemic H1N1/2009, which is reverse zoonotic event. The genetic analysis of CoV showed that canine respiratory coronaviruses (CRCoVs) were grouped into betacoronavirus which closely related to human CoV (HCoV-OC43) and bovine CoV. Thai CRCoVs were different from canine enteric coronaviruses of the genus alphacoronavirus. The TMRCA analysis indicated that Thai CRCoV was estimated to separate from HCoV-OC43 and BCoV with the most recent common ancestor since 2004. The results of the fourth to seventh topic were the surveillance of gastroenteric viruses in dogs including Kobuvirus (KoV), Norovirus (NoV), Rotavirus (RoV) and Canine parvovirus type 2 (CPV-2). These viruses can cause gastroenteric disease. Since some viruses have been reported zoonotic transmission. The rectal swab samples were collected from dogs with gastroenteritis signs. The occurrence of KoV, NoV, CPV-2, and RoV in dogs were 17.6% (54/307), 11.1% (2/18), 29.9% (133/444) and 0.7% (5/710), respectively. The genetic analysis of KoV in dogs showed that Thai-KoVs were closely related to KoV from China. The genetic analysis of NoV showed that Thai-NoVs in dogs belonged to genotype GII.Pe-GII.4 Sydney which is the common genotype causing NoV outbreaks in humans in Thailand. In this study, canine NoVs were detected from dogs living on the same premises with the confirmed human NoV case suggesting human-to-canine transmission. The genetic analysis of CPV-2, this study is the first report of CPV-2c in dogs and cats in Thailand. The genetic analysis of RoV showed that the genotype G3P[3] was a predominant genotype of RoV in dogs in Thailand. The pattern of genetic constellation of Thai RoVs was G3-P[3]-I3-R3-C3-M3-A9-N2-T3-E3-H6, which never been reported in dogs. The TMRCA analysis showed that Thai-RoV was estimated to separate from bat, human, and dog RoVs and subsequently generating novel RoV G3P[3]. The result of the eighth topic was the surveillance of respiratory and enteric viruses in human. Nasal swab, stool samples and questionnaire interview were obtained from 100 participants who had high-risk occupations and in close contact with dogs. The nasal swab samples were tested for influenza virus, parainfluenza virus and coronavirus, while stool samples were tested for coronavirus and rotavirus. The result of this study showed that CoV could be detected from two participants, while none of the other viruses (IAV, PIV and RV) could be detected. The genetic analysis of CoV showed that the human CoV belonged to alphacoronavirus of HCoV- 229E. The questionnaire interview showed that 52% of workers reported that they concern about the risk of zoonotic infection from dogs. Only 8% of participants reported using of gloves when working/contracting with sick dogs. However, this study involved a relatively small population with on one time sample collection. Therefore, a large population should be performed. The conclusion of this thesis, there are potential zoonotic respiratory and enteric viruses circulating in dogs in Thailand. Moreover, the genetic analysis of the viruses indicated that the viruses are rapid evolving especially after introduction of novel virus in the population and/or interspecies transmission. The information will be useful for people who have high-risk occupations such as veterinarians, vet assistants and owners. Moreover, these results provide information of the status, distribution, genetic characteristics of the viruses for the effective prevention and control of respiratory and enteric zoonotic viruses in dogs in Thailand.

Field of Study: Veterinary Public Health

Student's Signature .....

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Advisor's Signature .....

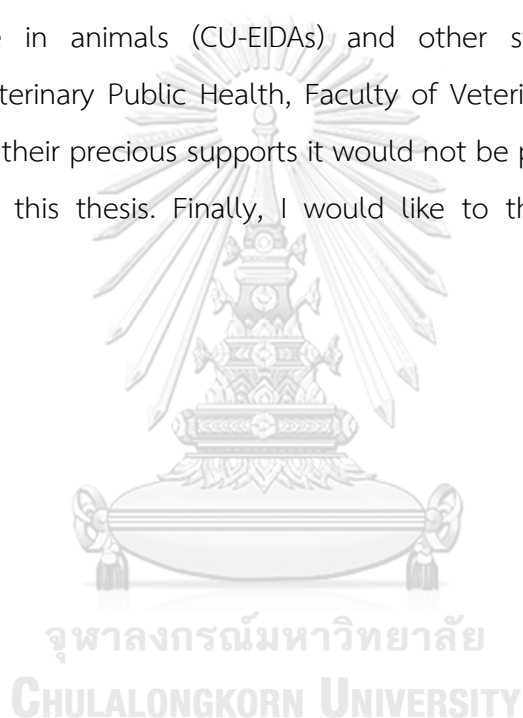
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## TABLE OF CONTENTS

	Page
ABSTRACT (THAI).....	iii
ABSTRACT (ENGLISH).....	iv
ACKNOWLEDGEMENTS .....	v
TABLE OF CONTENTS .....	vi
LIST OF TABLES .....	xii
LIST OF FIGURES .....	xvi
LIST OF ABBREVIATIONS .....	xxiii
CHAPTER I Introduction .....	1
1.1. Importance and Rationale.....	1
1.2 Objectives .....	4
1.3. Research questions.....	4
1.4. Literature Review.....	5
1.4.1. Canine parainfluenza virus type 5 (CPiV5).....	5
1.4.2. Canine influenza A virus (CIV).....	6
1.4.3. Canine respiratory coronavirus (CRCoV).....	7
1.4.4. Kobuvirus (KoV) .....	8
1.4.5. Norovirus (NoV).....	8
1.4.6. Canine parvovirus (CPV).....	9
1.4.7. Rotavirus (RV).....	10
CHAPTER II Canine Parainfluenza type 5 .....	13
2.1 Abstract .....	13

2.2 Introduction .....	14
2.3 Materials and Methods .....	16
2.3.1. Canine samples .....	16
2.3.2. Canine parainfluenza virus identification .....	16
2.3.3. Canine parainfluenza virus isolation .....	17
2.3.4. Canine parainfluenza virus characterization.....	17
2.3.5. Statistical analysis .....	18
2.4 Results .....	20
2.4.1. Genetic characteristics of Thai canine parainfluenza type 5 .....	22
2.5 Discussion.....	34
2.6 Conclusion .....	36
CHAPTER III Canine influenza virus.....	37
3.1 Abstract .....	37
3.2. Introduction.....	38
3.3. Materials and Methods Canine Samples .....	40
3.3.1 Dog samples.....	40
3.3.2 Influenza A Virus identification.....	40
3.3.3. Influenza A virus characterization.....	41
3.4 Results .....	44
3.4.1. Genetic characteristics of influenza A virus from dogs .....	46
3.4.2. Genetic analysis of Thai pdmH1N1-2009 (AD105).....	57
3.5. Discussion.....	60
3.6 Conclusion .....	62
CHAPTER IV canine respiratory coronaviruses (CRCoV).....	63

4.1 Abstract .....	63
4.2 Introduction .....	64
4.3. Materials and Methods .....	66
4.3.1. Sample collection from dogs .....	66
4.3.2. Identification of canine respiratory coronavirus (CRCoV).....	66
4.3.3. Characterization of canine respiratory coronavirus (CRCoV).....	66
4.4. Result.....	68
4.4.1. Canine Respiratory Coronavirus (CRCoV) in domestic dogs .....	68
4.4.2. Genetic characteristics of Thai CRCoV .....	71
4.4.3. Genetic analysis of Thai CRCoVs.....	82
4.5. Discussion.....	86
4.6. Conclusion.....	88
CHAPTER V Canine Kobuvirus.....	89
5.1 Abstract .....	89
5.2 Introduction.....	91
5.3 Material and Methods .....	92
5.3.1. Sample collection.....	92
5.3.2. Canine Kobuvirus (CaKoV) detection .....	92
5.3.3. Canine Kobuvirus characterization .....	93
5.3.4. Phylogenetic and genetic analyses of Canine Kobuviruses .....	95
5.4 Results .....	96
5.4.1. Canine Kobuviruses in domestic dogs in Thailand .....	96
5.4.2. Phylogeny of the Thai Canine Kobuviruses.....	101
5.4.3. Genetic analysis of the Thai Canine Kobuviruses.....	105

5.5 Discussion.....	110
5.6 Conclusion .....	112
CHAPTER VI Norovirus .....	113
6.1 Abstract .....	113
6.2 Introduction.....	114
6.3 Material and method.....	115
6.3.1. Infection in Dogs .....	115
6.3.2. Identification of Viruses .....	115
6.3.3. Characterization of norovirus .....	118
6.4 Results and discussion .....	120
6.5 Conclusion .....	132
CHAPTER VII Canine parvovirus type 2c.....	133
7.1 Abstract .....	133
7.2 Introduction.....	135
7.3 Material and methods.....	136
7.4. Results.....	141
7.5. Discussion.....	156
CHAPTER VIII Canine rotavirus .....	158
8.1 Abstract .....	158
8.2 Introduction.....	159
8.3 Materials and Methods .....	161
8.3.1. Sample collection.....	161
8.3.2. Canine rotavirus detection.....	161
8.3.3. Canine rotavirus characterization .....	162



8.4 Results .....	164
8.4.1. Canine rotavirus in dogs in Thailand.....	164
8.4.2. Genotype and the genetic constellation of Thai canine rotaviruses .....	164
8.4.3. Genotype G3P[3] of the Thai Canine Rotaviruses .....	169
8.4.4. Genetic analysis of Thai canine rotaviruses.....	183
8.5 Discussion.....	186
8.6 Conclusion .....	190
CHAPTER IX .....	191
9.1. Abstract.....	191
9.2. Introduction.....	193
9.3. Materials and Methods .....	195
9.3.1 Human sample collection.....	195
9.3.2. Questionnaire.....	195
9.3.3. Human coronavirus identification.....	196
9.3.4. Coronavirus characterization .....	196
9.4. Results .....	197
9.5. Discussion.....	211
9.6. Conclusion .....	212
CHAPTER X Conclusions and Recommendations .....	213
APPENDIX A .....	220
APPENDIX B .....	232
APPENDIX C .....	244
APPENDIX D.....	249
APPENDIX E .....	261

REFERENCES ..... 275

VITA..... 303



## LIST OF TABLES

### CHAPTER 2

Table 2.1. Nucleotide sequences of primers used for CPIV-5 detection and sequencing in this study.....	19
Table 2.2. Details of the sample collection and detection of CPIV- 5 .....	21
Table 2.3. Description of canine parainfluenza type 5 (CPIV-5) characterized in this study.....	24
Table 2.4. Pairwise comparison of whole genome nucleotide sequences of Thai CPIV-5 (CU-D151) with reference parainfluenza viruses.....	25
Table 2.5. Genetic analysis of the HN gene of Thai CPIV-5 and reference PIV-5 at the receptor binding, cleavage site and stalk region.....	27
Table 2.6. Genetic analysis of the HN gene of Thai CPIV-5 and reference PIV-5 at the human-specific residues.....	28
Table 2.7. Genetic analysis of the F gene of Thai CPIV-5 and reference PIV.....	29
Table 2.8. Genetic analysis of the V/P, SH and non-coding region of Thai CPIV-5 and reference PIV-5.....	30

### CHAPTER 3

Table 3.1. Description of influenza A virus detected and characterized in this study.	43
Table 3.2. Detail of samples collection and detection of influenza A virus.....	45
Table 3.3. Genetic constellation of Thai pdmH1N1-2009 (AD105) from dog.....	55
Table 3.4. Pairwise comparisons of nucleotide and amino acid of Thai pdmH1N1-2009 (AD105) from dog with those of reference influenza viruses.....	56
Table 3.5. Genetic analysis of the H1 gene of Thai pdmH1N1-2009 (AD105) from dog.....	58
Table 3.6. Genetic analysis of the internal genes of Thai pdmH1N1-2009 (AD105) from dog.....	59

## CHAPTER 4

Table 4.1. Associations between age of CRCoVs in this study.....	70
Table 4.2. Description of canine respiratory coronaviruses detected in this study.....	72
Table 4.3. Detail description of canine respiratory coronaviruses (CRCoVs) characterized this study.....	74
Table 4.4. Pair-wise sequence comparison of whole genome sequences of Thai-CRCoV and reference betaoronaviruses (CRCoV, BCoV, HCoV-OC43, HCoV-4408, SARS-CoV, SARS-CoV-2, and MERS) and alphacoronaviruses (CCoV, FCoV, HCoV-229E, PED, and TGEV) coronavirus from the GenBank database. ....	75
Table 4.5. Genetic analysis of HE gene of Thai-CRCoVs and reference etacoronaviruses (CRCoV, HCoV-OC43, BCoV, HCoV-4408).....	83
Table 4.6. Genetic analysis of S gene of Thai-CRCoVs and reference betacoronaviruses (CRCoV, BoV, HCoV-OC43 and HCoV-4408).....	84
Table 4.7. Genetic analysis of S gene of Thai-CRCoVs and reference CRCoVs from the GenBank.....	85

## CHAPTER 5

Table 5.1. Oligonucleotide primers used for CaKoV whole genome sequencing.....	94
Table 5.2. Association of age of CaKoVs detection in this study.....	99
Table 5.3. Detail description of Thai CaKoVs characterized in this study.....	100
Table 5.4. Pairwise comparison of whole genome of Thai CaKoVs (CU-101) and reference CaKoVs.....	106
Table 5.5. Pairwise comparison of 3D and VP1 genes of Thai CaKoVs (CU-101) and reference CaKoVs.....	107
Table 5.6. Genetic analysis of Thai CaKoVs compared with reference CaKoVs at proline rich region .....	108
Table 5.7. Genetic analysis of Thai CaKoVs compared with reference CaKoVs at putative amino acid cleavage sites.....	109

## CHAPTER 6

Table 6.1. List of primers for identification and sequencing of noroviruses, Thailand .....	117
Table 6.2 Characteristics of samples collected and examined from a dog kennel, Thailand, 2018** .....	123
Table 6.3. Detail description of Thai NoVs characterized in this study.....	126
Table 6.4. Genetic analysis of nucleotide sequences of canine and human noroviruses from Thailand for antigenic epitopes (A–E) major capsid protein compared with those for other noroviruses* .....	127
Table 6.5. Pairwise comparisons of nucleotides and amino acids of canine norovirus CU21939 from Thailand with those of reference noroviruses* .....	128

## CHAPTER 7

Table 7.1. List of primers for CPV2 typing and whole genome sequencing. ....	140
Table 7.2. Association between age and clinical presentations of CPV-2 and FPV detection in this study.....	141
Table 7.3. Association between vaccine history and clinical presentations of CPV-2 and FPV detection in this study .....	142
Table 7.4. Detail of sample tested and CPV2 variants in this study.....	144
Table 7.5. Detailed descriptions of CPV-2 and FPV characterized in this study.....	145
Table 7.6. Pairwise comparisons of whole genome of Dog/Thailand/CU-24 and Cat/Thailand/CU-21 with those of reference CPV and FPV.....	146
Table 7.7. Pairwise comparisons of partial VP2 gene of Dog/Thailand/CU-24 with Thai- CPV and FPV and reference CPV and FPV.....	147
Table 7.8. Genetic analysis of deduced amino acids of Thai-CPV-2 and FPV in comparison to those of vaccine and reference strains .....	153

## CHAPTER 8

Table 8.1. Description of the Thai CRVs characterized in this study. ....	165
---	-----

Table 8.2. Association between age and clinical presentations of CRVs detection in this study.....	165
Table 8. 3. The genetic constellation of the Thai CRVs and reference rotaviruses....	166
Table 8.4. Pair-wise comparison among genes of Thai CRVs and reference rotaviruses. ....	167
Table 8.5. Nucleotide identities, genotype, nucleotide substitution rate and 95% posterior densities of each gene of the Thai CRVs.....	171
Table 8.6. Genetic analysis of antigenic regions at VP7 among the Thai CRVs and reference RVAs from dogs, cats, bats, humans, and vaccine strain.....	184
Table 8.7. Amino acid analysis of G3 between Thai CRVs and reference RVAs from dogs, cats, bats, humans representing cluster A, B and sub-cluster b1 and b2. ....	185
<b>CHAPTER 9</b>	
Table 9.1. Detail description of human coronaviruses (HCoV) characterized this study. ....	198
Table 9.2. Pairwise comparison of nucleotide sequences of RdRp gene of Thai HCoV and reference coronaviruses.....	199
Table 9.3. Demographic characteristics of high-risk occupations who have been in close contacted with sick dogs (n=100).....	202
Table 9.4 Information of small animal hospital management regarding to zoonotic diseases.....	203
Table 9.5. Practices of persons who have been in close contact with dogs with gastroenteritis signs. ....	205
Table 9.6. Practices of persons who have been in close contact with dogs with respiratory signs.....	206
Table 9.7. Summary of knowledge, attitudes, and experience of participants in this study.....	208

## LIST OF FIGURES

### CHAPTER 2

- Figure 2.1. Phylogenetic tree of whole genome of Thai CPIV-5 and reference PIV1-5. Pink circles indicate Thai CPIV-5 in this study..... 31
- Figure 2.2. Phylogenetic trees of the HN, F, M, V/P, NP, and L genes of Thai CPIV-5 and reference PIV-1-5. Pink circles indicate Thai CPIV-5 in this study..... 32
- Figure 2.3. Alignment of deduced amino acids of SH gene of Thai CPIV-5 and reference PIV-5. The box indicates amino acid substitution at the stop codon (Q). Pink circles indicate Thai CPIV-5 in this study..... 33

### CHAPTER 3

- Figure 3.1. Phylogenetic tree of H1 gene. The phylogenetic tree was constructed by using the neighbor-joining algorithm with kimura-2 parameter model with 1000 replication bootstrap. The pink symbol indicates Thai pdmH1N1-2009 in this study. The blue triangular are feline pdmH1N1-2009 from cats. The green triangular are pdmH1N1-2009 from dogs..... 47
- Figure 3.2. Phylogenetic tree of N1 gene. The phylogenetic tree was constructed by using the neighbor-joining algorithm with kimura-2 parameter model with 1000 replication bootstrap. The pink symbol indicates Thai pdmH1N1-2009 in this study. The blue triangular are feline pdmH1N1-2009 from cats. The green triangular are pdmH1N1-2009 from dogs..... 48
- Figure 3.3. Phylogenetic tree of M gene. The phylogenetic tree was constructed by using the neighbor-joining algorithm with kimura-2 parameter model with 1000 replication bootstrap. The pink symbol indicates Thai pdmH1N1-2009 in this study. 49
- Figure 3.4. Phylogenetic tree of NP gene. The phylogenetic tree was constructed by using the neighbor-joining algorithm with kimura-2 parameter model with 1000 replication bootstrap. The pink symbol indicates Thai pdmH1N1-2009 in this study. The blue triangular are feline pdmH1N1-2009 from cats. The green triangular are pdmH1N1-2009 from dogs..... 50

Figure 3.5. Phylogenetic tree of NS gene. The phylogenetic tree was constructed by using the neighbor-joining algorithm with kimura-2 parameter model with 1000 replication bootstrap. The pink symbol indicates Thai pdmH1N1-2009 in this study. The blue triangular are feline pdmH1N1-2009 from cats. The green triangular are pdmH1N1-2009 from dogs..... 51

Figure 3.6. Phylogenetic tree of PA gene. The phylogenetic tree was constructed by using the neighbor-joining algorithm with kimura-2 parameter model with 1000 replication bootstrap. The pink symbol indicates Thai pdmH1N1-2009 in this study. The blue triangular are feline pdmH1N1-2009 from cats. The green triangular are pdmH1N1-2009 from dogs..... 52

Figure 3.7. Phylogenetic tree of PB1 gene. The phylogenetic tree was constructed by using the neighbor-joining algorithm with kimura-2 parameter model with 1000 replication bootstrap. The pink symbol indicates Thai pdmH1N1-2009 in this study. The blue triangular are feline pdmH1N1-2009 from cats. The green triangular are pdmH1N1-2009 from dogs..... 53

Figure 3.8. Phylogenetic tree of PB2 gene. The phylogenetic tree was constructed by using the neighbor-joining algorithm with kimura-2 parameter model with 1000 replication bootstrap. The pink symbol indicates Thai pdmH1N1-2009 in this study. The blue triangular are feline pdmH1N1-2009 from cats. The green triangular are pdmH1N1-2009 from dogs..... 54

#### CHAPTER 4

Figure 4.1. Percentage of CRCoVs positivity in dogs in Bangkok Thailand by months during NoV 2015- Dec 2018..... 69

Figure 4.2. Phylogenetic tree based on whole genome sequence of Thai-CRCoVs and reference alphacoronaviruses and betacoronaviruses. The phylogenetic tree was constructed by using MEGA v.7.0 with neighbor-joining algorithm with kimura-2 parameter model and bootstrap analysis of 1,000 replications. Pink circle indicates Thai CRCoV in this study. .... 77



Figure 4.3. Phylogenetic tree based on HE gene of Thai-CRCoV and reference alphacoronaviruses and betacoronaviruses. The phylogenetic tree was constructed by using MEGA v.7.0 with neighbor-joining algorithm with kimura-2 parameter model and bootstrap analysis of 1,000 replications. Pink circle indicates Thai CRCoV in this study..... 79

Figure 4.4. The maximum clade credibility (MCC) tree base on S gene of Thai-CRCoV and reference alphacoronaviruses and betacoronaviruses. The phylogenetic tree was constructed by using BEAST 1.10 with Baysian Markov Chain Monte Carlo (BMCMC) algorithm. A strict clock model with a coalescent population and GTR (I)substitution were used as model parameters. Pink text indicated Thai-CRCoV characterized in this study..... 80

Figure 4.5. Phylogenetic tree based on S gene of Thai-CRCoV and reference alphacoronaviruses and betacoronaviruses. The phylogenetic tree was constructed by using MEGA v.7.0 with neighbor-joining algorithm with kimura-2 parameter model with bootstrap analysis of 1,000 replications. Pink circle indicates Thai-CRCoV in this study..... 81

## CHAPTER 5

Figure 5.1. Number of samples collected and CaKovs detected by month in this study..... 97

Figure 5.2. Number of samples collected and CaKovs detected by provinces in this study (Map of Thailand with permission by World Trade Press)..... 98

Figure 5.3. Phylogenic tree of the completed genome of CaKovs. The phylogenetic tree was constructed by using MEGA v6.0 with neighbor-joining algorithm with Kimura-2 parameter model and Beast program with Bayesian Markov chain Monte Carlo (BMCMC) with 10,000,000 generations and an average standard deviation of split frequencies <0.05. Values on branches represent bootstrap and posterior probability values. .... 102

- Figure 5.4. Phylogenetic tree of 3D gene of CaKoVs. The phylogenetic tree was constructed by using MEGA v 6.0 with neighbor-joining algorithm with Kimura-2 parameter model with 1,000 replications of bootstrap analysis..... 103
- Figure 5.5. Phylogenetic tree of VP1 gene of CaKoVs. The phylogenetic tree was constructed by using MEGA v6.0 with neighbor-joining algorithm with Kimura-2 parameter model with 1,000 replications of bootstrap analysis..... 104

## CHAPTER 6

- Figure 6.1. Human norovirus infection in dogs, Thailand. A) Diarrhea. B) Collection of fecal samples. .... 121
- Figure 6.2. Phylogenetic tree of whole-genome sequences of canine noroviruses (red dots) and human noroviruses (blue triangles) from Thailand and reference sequences. Genogroups GI–GVII are indicated by red oval and blue ovals. The tree was constructed by using MEGA version 7.026 (<https://www.megasoftware.net>) with the neighbor-joining algorithm and bootstrap analysis with 1,000 replications. Numbers along branches are bootstrap values. Scale bar indicates nucleotide substitutions per site..... 129
- Figure 6.3. Phylogenetic tree of open reading frame 1 of canine noroviruses (purple dots) and human noroviruses (blue squares) from Thailand and reference sequences. Tree was constructed by using MEGA version 7.026 (<https://www.megasoftware.net>) with the neighbor-joining algorithm and bootstrap analysis with 1,000 replications. Numbers along branches are bootstrap values, and numbers on the right indicate genogroups. Scale bar indicates nucleotide substitutions per site..... 130
- Figure 6.4. A) Phylogenetic tree of ORF2 of noroviruses. B) Phylogenetic tree of ORF2 of GI.4 noroviruses. Red circles indicate canine noroviruses from Thailand, green triangles indicate canine noroviruses from Finland, and blue squares indicate human noroviruses from Thailand. Trees were constructed by using MEGA version 7.026 (<https://www.megasoftware.net>) with the neighbor-joining algorithm and bootstrap analysis with 1,000 replications. Numbers along branches are bootstrap values, and

numbers on the right of panel A indicate genogroups. Scale bars indicate nucleotide substitutions per site. ORF, open reading frame..... 131

## CHAPTER 7

Figure 7.1. Phylogenetic tree of VP2 gene of canine parvovirus type 2 and feline parvovirus. Circles and squares represent Thai-CPV-2 and FPV, respectively. The phylogenetic tree was constructed by using the Beast program with Bayesian Markov-Chain Monte Carlo (BMCMC), with 10,000,000 generations and an average standard deviation of split frequencies ..... 150

Figure 7.2. Phylogenetic tree of VP2 gene sequences of the canine parvovirus type 2 (CPV-2a, CPV-2b, CPV-2c, CPV-2a-297A, CPV-2b-297A) and feline parvovirus. Thai-CPV-2 and FPV (n=34) are included in the phylogenetic tree. Circles and triangles represent Thai- CPV-2 and FPV, respectively. Squares represent CPV and FPV vaccine strains. Phylogenetic tree was constructed by using MEGA v.6.06 program with neighbor-joining algorithm applying bootstrap analysis with 1,000 replications..... 151

Figure 7.3. Amino acid alignment of VP2 protein of CPV-2. Dots represent matched amino acid residues. Open boxes indicate amino acid substitutions..... 155

## CHAPTER 8

Figure 8.1. Phylogenetic tree of VP7. The maximum clade credibility (MCC) tree was constructed by BEAST 1.8 with the Bayesian Markov Chain Monte Carlo (BMCMC) algorithm. A strict clock model with a coalescent constant population and GTR, TN93 (G+I) and HKY with a gamma 4 substitution were used as model parameters. Pink circles indicate the Thai CRVs characterized in this study. Dog images (orange) represent the Thai CRVs and human images (black) represent reference human RVs closely related to the Thai CRVs..... 172

Figure 8.2. Phylogenetic tree of VP4. The maximum clade credibility (MCC) tree was constructed by BEAST 1.8 with the Bayesian Markov Chain Monte Carlo (BMCMC) algorithm. A strict clock model with a coalescent constant population and GTR, TN93 (G + I) and HKY with a gamma 4 substitution were used as model parameters. Pink circles indicate the Thai CRVs characterized in this study. Dog images (orange)

represent the Thai CRVs and bat images (black) represent the reference bat RVs closely related to the Thai CRVs.....	173
Figure 8.3. Phylogenetic tree of VP6. The maximum clade credibility (MCC) tree was constructed by BEAST 1.8 with the Bayesian Markov Chain Monte Carlo (BMCMC) algorithm. A strict clock model with a coalescent constant population and GTR, TN93 (G+I) and HKY with a gamma 4 substitution were used as model parameters. Pink circles indicate the Thai CRVs characterized in this study. Dog images (orange) represent the Thai CRVs and human images (black) represent the reference human RVs closely related to the Thai CRVs. ....	174
Figure 8.4. Phylogenetic tree of VP1 of Rotaviruses. Circles represent Thai CRVs characterized in this study.....	175
Figure 8.5. Phylogenetic tree of VP2 of Rotaviruses. Circles represent Thai CRVs characterized in this study.....	176
Figure 8.6. Phylogenetic tree of VP3 of Rotaviruses. Circles represent Thai CRVs characterized in this study.....	177
Figure 8.7. Phylogenetic tree of NSP1 of Rotaviruses. Circles represent Thai CRVs characterized in this study.....	178
Figure 8.8. Phylogenetic tree of NSP2 of Rotaviruses. Circles represent Thai CRVs characterized in this study.....	179
Figure 8.9. Phylogenetic tree of NSP3 of Rotaviruses. Circles represent Thai CRVs characterized in this study.....	180
Figure 8.10. Phylogenetic tree of NSP4 of Rotaviruses. Circles represent Thai CRVs characterized in this study.....	181
Figure 8.11. Phylogenetic tree of NSP5 of Rotaviruses. Circles represent Thai CRVs characterized in this study.....	182
Figure 8.12. Schematic presentation of possible multiple-reassortment of the Thai CRVs among the dog, bat, and human rotaviruses. ....	189

**CHAPTER 9**

Figure 9.1. Phylogenetic tree of Thai HCoV based on RdRp gene. The phylogenetic tree was constructed by using MEGA v.7.0 with neighbor-joining algorithm with kimura-2 parameter model with bootstrap analysis of 1,000 replications. Blue squares presented HCoV in this study. Pink circulars presented canine CoV (CRCoV) in this study.....210



## LIST OF ABBREVIATIONS

bp	Base pair(s)
cDNA	Complementary deoxyribonucleic acid
CaKoV	Canine kobuvirus
CIV	Canine influenza virus
CPIV-5	Canine parainfluenza virus type 5
CCoV	Canine coronavirus
CPV-2	Canine parvovirus type 2
CRCoV	Canine respiratory coronavirus
CRV	Canine rotavirus
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
et al.	Et alibi, and others
FAM	Carboxyfluorescein
g	Gram(s)
M	Molar
MgCl <sub>2</sub>	Magnesium chloride
mL	Milliliter(s)
mM	Millimolar
NoV	Norovirus

PBS	Phosphate-buffer saline
PCR	Polymerase chain reaction
PIV	Parainflueza virus
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
rpm	Round per minute
WGS	Whole genome sequencing
°C	Degree Celsius
µg	Microgram(s)
µL	Microliter(s)
µM	Micromolar

The parts of this dissertation have been published in 5 international journals including;

1. Emergence of canine parvovirus type 2c in domestic dogs and cats from Thailand” in *Transboundary and Emerging Disease*; 2019 Jul;66(4):1518-1528.
2. First Detection and Genetic Characterization of Canine Kobuvirus in domestic dogs in Thailand” in *BMC Veterinary Research*; 15, Article number: 254 (2019)
3. Human Norovirus Infection in Dogs, Thailand” in *Emerging Infectious Disease*; 2020;26(2):350-353.
4. Molecular characterization identifies intra-host recombination and zoonotic potential of canine rotavirus among dogs from Thailand” in *Transboundary and Emerging Disease*; 2020 Aug 9. doi: 10.1111/tbed.13778.
5. Molecular detection and whole genome characterization of Canine Parainfluenza type 5 in Thailand” in *Scientific Reports*, 11, Article number: 3866 (2021)

The parts of this dissertation have been published in poster presentations.

1. Occurrence of canine parainfluenza virus type 5 in Thailand. The 16<sup>th</sup> Chulalongkorn University Veterinary Conference – CUVC 2017, 22-24 March 2017 Bangkok, Thailand.
2. A. Occurrence of canine respiratory coronavirus in Thailand. The 17<sup>th</sup> Chulalongkorn University Veterinary Conference – CUVC 2018, 25-27 April 2018 Bangkok, Thailand.
3. Canine-Human Interface, risk of Canine Rotavirus strain G3P[3] in Thailand. The 3<sup>rd</sup> International Conference on One Medicine One Science – ICOMOS 2018, 29 April – 2 May 2018 Minnesota, USA.
4. Genetic characterization of canine respiratory coronaviruses in Thailand, during 2016-2017. 43<sup>RD</sup> world small animal veterinary association congress and 9<sup>TH</sup> FASAVA congress – WSAVA 2018, 25-28 September 2018, Singapore.



**The 3 manuscripts in this dissertation are in preparation including;**

1. Characterization of pandemic H1N1-2009 in dogs, Thailand
2. Genetic diversity of canine respiratory coronaviruses (CRCoV) in dogs, Thailand
3. Surveillance of potential respiratory and enteric zoonotic viruses in High-risk occupations in Bangkok, Thailand which the manuscript is in preparation.



## CHAPTER I INTRODUCTION

### 1.1. Importance and Rationale

Human-Dog interface is one of the factors contributing to the risk of zoonotic diseases. Dogs are considered as the reservoirs and/or carriers of several potential zoonotic viruses such as rabies virus, influenza virus, parainfluenza virus, coronavirus, and rotavirus etc. These viruses can circulate among dogs and possible transmit to human via saliva, respiratory droplet, secretion, feces, and fomite (Chen et al., 2012; De Grazia et al., 2007b; Song et al., 2015b).

The potential zoonotic respiratory viruses in dogs include influenza A virus (IAV), parainfluenza virus type 5 (PIV5) and coronavirus (CoV). Influenza A virus (IAV) is one of the major causes of respiratory illness in animals and humans worldwide (Webster et al., 1992). Canine influenza virus (CIV) leads to outbreaks and endemics of canine respiratory diseases in several countries including USA, China, Korea, and Thailand (Bunpapong et al., 2014b; Crawford et al., 2005a; Song et al., 2012a). Since dogs are susceptible for influenza A virus infection of both avian and human-origins, dogs play an important role as an intermediate host for generating novel or reassorted influenza viruses (Song et al., 2012a). To date, there is no report of CIV transmission from dogs to humans, however potential zoonotic transmission of CIV should not be ignored. Parainfluenza virus type 5 (PIV5) is also considered as important zoonotic pathogen. It can infect widely host ranges including humans, pigs, and dogs (Chatziandreou et al., 2004). Canine parainfluenza virus type 5 (CPIV5) is one of the common pathogens of canine infectious respiratory disease (CIRD). Several publications reported that CPIV5 could be a potential zoonotic virus associated with multiple sclerosis and respiratory disease in humans (Chen et al., 2012; Goswami et al., 1987b). Canine respiratory coronavirus (CRCoV) is common cause of respiratory diseases in dogs. Infected dogs have mild to severe respiratory diseases including cough, sneezing, nasal, and ocular discharge. The genetic characteristics of CRCoV are similar to human coronavirus (HCoV-OC43) and bovine coronavirus (BCoV) (Vijgen et al., 2005).

The important enteric viruses in dogs are Kobuvirus, parvovirus, norovirus, and rotavirus. Norovirus and rotaviruses cause acute gastroenteritis in animal and human and lead to half of million deaths annually (Parashar et al., 2009). Noroviruses are species specific and only subtype GI, GII and GIV cause disease in human (Vinje, 2015). However, cross species transmission between human and animal have been reported worldwide. Rotavirus are several subtypes causing enteric disease in humans and animals, but G3P[3] and G3P[9] strains are predominant strains in dogs and cats (Martella et al., 2010). The G3P[3] and G3P[9] strains are one of the public health concerns as zoonotic potential, although limited data has been observed in human (Matthijnssens et al., 2011b). In Thailand, human rotavirus outbreaks has been periodic reported and rare strains G3P[3] and G3P[9] in gastroenteric illness in children has also been reported (Khamrin et al., 2006a; Theamboonlers et al., 2013a). Another important enteric virus in dogs are canine kobuvirus (CaKoV) and canine parvovirus type 2 (CPV2). Nowadays, there is no strong evidence that viruses are a potential zoonotic disease, but they are important pathogen causing hemorrhagic gastroenteritis and death in puppy worldwide. Especially, CPV2 has a high mutation rate, which can generate new pandemic variants such as CPV-2a, 2b, and 2c (Mohan Raj et al., 2010). Moreover, CPV2 could co-infect and influence to zoonotic pathogens outbreaks with other zoonotic viruses such as rotavirus (Ortega et al., 2017a).

In Bangkok, human population lives in approximately 6 million according to data from National Statistical Office in 2016, and the number of dog population is almost a million (both registered and stray dogs) as reported by Bureau of Disease Control and Veterinary Services, 2016. Dog-human interface is common in such high-density area. It could be one of the factors to elevate the risk of zoonotic diseases. Moreover, people with high-risk occupations (veterinary practitioners, animal care workers, etc.) are more likely to infect and spread zoonotic pathogens from dogs (Baker and Gray, 2009). In Thailand, the data of canine respiratory (CIV, CPiV5, and CRCoV) and enteric (Kobuvirus, Norovirus, CPV2, and Rotavirus) viruses are still limited. Particularly, canine rotavirus and norovirus have never been identified in

Thailand. Therefore, the monitoring of these viruses in both humans and dogs is essential for disease prevention and control. Thus, survey on canine respiratory and enteric viruses from dogs and humans in Bangkok was conducted during January 2016 - June 2018. This study was also elucidated genetic characteristics of potential canine respiratory and enteric zoonotic viruses. The prevention, control strategies and risk communications of potential canine respiratory and enteric zoonotic viruses was explained.



## 1.2 Objectives

Due to the importance public health concern and limitation data of human-dog viruses, this study was conducted these viruses in both humans and dogs for disease prevention and control.

### **The objectives of this study were:**

1. To determine the occurrence of potential zoonotic canine respiratory and enteric viruses
2. To determine the genetic characteristics of canine respiratory and enteric viruses
3. To determine the protective factors of veterinarians and animal care workers on exposure to potential zoonotic canine viruses in animal hospitals in Bangkok, Thailand

## 1.3. Research questions

### **Research questions of this study were:**

1. What are the occurrence of potential zoonotic canine respiratory and enteric viruses in dogs in Bangkok, Thailand?
2. What are the major strains/subtypes of canine respiratory and enteric viruses circulating in Bangkok, Thailand?
3. What are the protective factors of veterinarians and animal care workers on exposure to potential zoonotic canine viruses in animal hospitals in Bangkok, Thailand?

#### 1.4. Literature Review

Human-dog interface is one of the factors contributing to the risk of zoonotic diseases. Dogs are considered as the reservoirs and/or carriers of several potential zoonotic viruses such as rabies virus, influenza virus, parainfluenza virus, coronavirus, and rotavirus etc. These viruses can circulate among dogs and possible transmit to human via saliva, respiratory droplet, secretion, feces, and fomite (Chen et al., 2012; De Grazia et al., 2007b; Song et al., 2015b). In this study, we were highlight on the importance respiratory and enteric viruses following

- Respiratory viruses
  - Canine parainfluenza virus type 5 (CPIV-5)
  - Canine influenza virus (CIV)
  - Canine respiratory coronavirus (CRCoV)
- Enteric viruses
  - Canine kobuvirus
  - Norovirus
  - Canine parvovirus type 2
  - Rotavirus group A

##### 1.4.1. Canine parainfluenza virus type 5 (CPIV5)

Parainfluenza virus (PIV) could be divided into 5 types as PIV 1-5. PIV 1 to 4 can cause upper and lower respiratory tract diseases in children (Morgan et al., 2013; Ruampunpong et al., 2014). While PIV5 is believed to cause a respiratory disease in dog. Some studies showed that PIV5 can also cause respiratory diseases in other mammals such as calf, swine, and human (Goswami et al., 1987b; Lee and Lee, 2013; Zhang et al., 2011). PIV5 is a single-stranded, non-segmented and enveloped negative-sense RNA virus. The virus consists of 7 genes which encoding 8 proteins (F, HN, SH, M, NP, V, P, and L). In 1956, PIV 5 was first described in monkey, which was called simian virus type 5 (SV5) (Hull et al., 1956). PIV5 was first reported in human in 1972 (Hsiung, 1972). The impact of PIV5 to human has not been explained. However, some studies revealed that PIV5 might be associated with respiratory disease and multiple sclerosis in human (Zhang et al., 2011). The study of genetic

characterization and animal experiment revealed that PIV5 might have been a potential zoonotic disease (Chen et al., 2012).

Canine Parainfluenza virus type 5 (CPIV5) was first isolated from dogs with a respiratory disease in 1967 (Binn et al., 1967). CPIV5 is one of the common causes of respiratory infectious diseases in dogs. Infected dogs will show mild to moderate respiratory illnesses including dry cough, fever, nasal discharge, and rarely neurological disorders (posterior paresis) (Baumgartner et al., 1981). CPIV5 is a highly contagious pathogen and has long shedding periods more than 10 days (Ellis and Krakowka, 2012). Apart from dogs, CPIV5 was also reported in ferret and cat (Black and Lee, 1970). In Thailand, CPIV5 was reported in dogs (11.93%) with respiratory symptoms (Posuwan et al., 2010a). However, the epidemiology and genetic information of CPIV5 in dogs in Thailand is still limited.

#### **1.4.2. Canine influenza A virus (CIV)**

Influenza virus has adverse effect on economy and public health worldwide. Influenza virus is negative sense, single-stranded RNA virus and belongs to the Orthomyxoviridae family. Influenza virus can be divided into 3 types (A, B and C). Influenza A is the most virulence in humans and animals. Influenza virus comprises with 8 genes encoding 11 proteins (HA, NA, NP, M1, M2, NS1, NS2, PA, PB1, PB1-F2 and PB2). Subtyping of influenza A virus depends on two surfaces envelop proteins, HA and NA. To date, 18 HA (H1-18) and 11 NA (N1-11) subtypes have been reported (Tong et al., 2013b).

Canine influenza virus (CIV) causes canine influenza outbreaks in dogs worldwide. In 2004, CIV subtype H3N8 (CIV-H3N8) of equine origin was first reported in racing greyhound dogs with respiratory disease in Florida, USA (Crawford et al., 2005a). Consequently, H1N1, H3N2 were reported in dogs in several countries (Lin et al., 2012b; Song et al., 2012a). Infected CIV dogs developed respiratory symptoms and possibly complications due to secondary bacterial infection including cough, nasal discharge, fever, and pneumonia. In Thailand, CIV subtypes H5N1 and H3N2 were reported in dogs with respiratory signs. The prevalence of CIV of dogs with respiratory diseases was also reported 2.75% (Posuwan et al., 2010a). Interestingly, a

serological survey indicated that dogs in Thailand had antibody against to both canine influenza (H3N2) and human influenza (H3N2, pH1N1) (Chanvatik et al., 2016).

Because canine respiratory tract is susceptible to influenza viruses of both avian- and human-origins, dogs could serve as “mixing vessel” for influenza virus and generate new subtypes or reassortment viruses. For example, a novel CIV-H3N1 is a reassortant between pandemic H1N1 2009 and CIV-H3N2 (Song et al., 2012a). Thus, close contact between dogs and humans will increase an opportunity of human exposure to CIV (Parrish et al., 2015b). Up to date, there are limited research on CIV in Thailand. The monitoring of IAV infections in dogs should be routinely conducted to provide strategic planning for influenza prevention and control in dogs in the future.

#### **1.4.3. Canine respiratory coronavirus (CRCoV)**

Coronavirus (CoV) is an enveloped positive stranded RNA virus. CoV belongs to the *Coronaviridae* family. This family is divided into four genera including alphacoronavirus, betacoronavirus, deltacoronavirus and gammacoronaviruses. Coronavirus structure contains difference proteins such as spike protein (S), membrane protein (M), envelop protein (E), haemagglutinin esterase protein (HE) and the nucleocapsid protein (N). The coronavirus has a high mutation rate and can infect in various host species. Some members of coronavirus have been emerged and posed the ability to cross-species transmission such as MERS-CoV and SARS-CoV (Woo et al., 2010b)

Dog is a susceptible host for coronavirus. Canine respiratory coronavirus (CRCoV) belongs to the genus *betacoronavirus*. The CRCoV is an emerging coronavirus and was first reported in dogs with severe respiratory illnesses in UK (Erles et al., 2003a). Then, CRCoVs were reported worldwide including Asia and Europe (An et al., 2010; Mitchell et al., 2013). Clinical signs of infected CRCoV dogs are mild to severe respiratory signs and occasionally fatal due to bronchopneumonia. CRCoVs has a long period shedding (>10 days) and highly contagious particularly in kennel dogs (Mitchell et al., 2013). Moreover, CRCoV is a predisposing cause of secondary bacterial infection (Priestnall et al., 2009).



Genetic characterization of CRCoV revealed that CRCoV is closely related to human coronavirus (HCoV-OC43) and bovine CoV (Erles et al., 2007). The study suggested that the viruses have common ancestor and have ability to cross-species transmission (Vijgen et al., 2005). In Thailand, human and bovine CoVs have been reported (Singasa et al., 2017; Soonnarong et al., 2016).

#### **1.4.4. Kobuvirus (KoV)**

Kobuvirus (KoV) is a single-strand positive-sense RNA virus. KoV belongs to the family *Picornaviridae*, genus *Kobuvirus*, which consists of four species Aichivirus A, B, C and D (Adams et al., 2016; Oem et al., 2014b; Yamashita et al., 2003). KoV has been reported in feces of several mammal species including humans, ruminants, pigs, dogs, cat, bat and rodents (Carmona-Vicente et al., 2013; Khamrin et al., 2009; Li et al., 2010a; Lu et al., 2018; Mohamed et al., 2018; Phan et al., 2011; Yamashita et al., 2003). The Kobuvirus species Aichivirus A contains four types including Aichi virus 1, Canine Kobuvirus 1 (CaKoV), Feline Kobuvirus 1 (FeKoV) and Murine Kobuvirus 1 (MuKoV). Canine Kobuvirus 1 (CaKoV) was first reported in dogs with acute gastroenteritis in the US in 2011 (Kapoor et al., 2011; Li et al., 2011). CaKoV was subsequently reported in dogs in UK, Italy, Australia, Japan, Korea and China (Carmona-Vicente et al., 2013; Di Martino et al., 2013; Kong et al., 2016; Oem et al., 2014a; Soma et al., 2016b). The virus was reported in wild carnivore (Jackal and Hyena) and domestic dogs in Tanzania, Africa (Olarte-Castillo et al., 2015), in foxes in Spain (Bodewes et al., 2014) and in foxes (Di Martino et al., 2014) and wolves in Italy (Melegari et al., 2018). Several studies have reported the detection of CaKoV infection in dogs with or without diarrhea and sometime systemic infection (Ribeiro et al., 2017). To date, only 12 completed CaKoV genomes are available in the GenBank database.

#### **1.4.5. Norovirus (NoV)**

Norovirus (NoV) infection is a major cause of both endemic and epidemic acute gastroenteritis. NoVs have been classified into 7 genogroups based on the VP1 major capsid protein. NoVs GI, GII and GIV can infect humans, NoVs GII can infect pigs, NoVs GIII and GV can infect ruminants and mice, and NoVs GVI and GVII can

infect dogs (Vinje, 2015). The evolutionary mechanisms of NoVs can be analyzed based on the recombination at the RNA-dependent RNA polymerase (RdRp) (ORF1) and the major capsid protein VP1 (ORF2) loci of the norovirus genome (Zheng et al., 2006). Newly emerged norovirus strains may lead to increasing incidence of norovirus infection worldwide. GII.4 is one of the predominant genogroup globally circulating genotypes. Genetic diversity of NoVs has been reported in a wide range of animals, such as pigs, cattle, and dogs.

Canine norovirus (CaNoV) was first reported as GIV.2 genotype in Italy in 2007 (Martella et al., 2008). Subsequently, the CaNoVs have been reported causing diseases in dogs in several countries in Asia and Europe (Caddy et al., 2013; Mesquita et al., 2010a; Mesquita and Nascimento, 2012; Ntafis et al., 2010). On the other hand, the seroprevalence of human noroviruses (HuNoVs) in dogs in the UK was reported as 13% (Caddy et al., 2013). Notably, GII.4 genotype (variants GII.4-2006b and GII.4-2008) was reported in dogs in Finland, indicating HuNoVs could transmit to and cause diarrhea in dogs (Summa et al., 2012). In humans, CaNoV antibodies were also reported in veterinarians, whose experience high risk exposure (Mesquita et al., 2013). However, there are only a few reports of HuNoV infection in dogs, and only limited numbers of complete CaNoV genomes are available in the GenBank database.

#### 1.4.6. Canine parvovirus (CPV)

Parvovirus (PV) belongs to the *Parvoviridae* family. PV is a non-enveloped, and single stranded DNA virus. PV contains two major open reading frames; the first ORF encoding two non-structural proteins (NS1, and NS2), and the second ORF encoding two viral capsid proteins (VP1, and VP2). Canine parvovirus type 2 (CPV-2) was first reported in gastroenteritis dogs in 1978. The genetic of CPV is similar to feline panleukopenia virus (FPLV) leading to hypothesis that FPLV was ancestor of CPV (Parrish, 1999). CPV-2 has a high substitution rate resulted in generating new pandemic variants such as CPV-2 strains 2a, 2b, and 2c (Mohan Raj et al., 2010). CPV-2a, 2b are major important strains in Asia and CPV-2c is a possible major strain in several countries especially in Europe (Decaro et al., 2005).

Clinical signs of CPV2 infected dogs are fever, anorexia, vomiting, mucous to hemorrhagic diarrhea, leukopenia and rarely subacute myocarditis. CPV2 also causes leuokopenia (lymphopenia and neutropenia), which leads to secondary infections (Ortega et al., 2017a). Target organs for CPV2 virus replication are intestinal and lymphoid organs. The virus is highly contagious, especially in puppy. Recently, CPV-2c was reported in severe gastroenteritis in adult vaccinated dogs (Decaro et al., 2008). Thus, the emerging of CPV-2c is a major concern on the effectiveness of CPV-2 commercial vaccines against CPV-2c (Calderon et al., 2009).

In Thailand, CPV2 is one of the major causes of infectious gastroenteritis disease. Up to date, CPV-2a is a major strain circulating while CPV-2c has not been reported (Phromnoi et al., 2010).

#### **1.4.7. Rotavirus (RV)**

Rotavirus is an important enteric pathogen in various hosts such as birds, humans, pigs, and dogs. Rotavirus belongs to the family *Reoviridae*, genus *Rotavirus*. Rotavirus is a non-enveloped, and double stranded RNA virus. The virus genome comprises with 11 segments, which encoding structural proteins (VP1-4, VP6-7) and non-structural proteins (NSP1-5). Rotavirus can be classified to A-G serogroup. On the other hand, Rotavirus can also be classified into genotype as GxPx; G (1-27), P (1-37) (Matthijnssens et al., 2011a). In general, group A rotavirus (GARV) causes diseases in both humans and animals and has the major impact on public health. GARV is causing more than half of million deaths in children annually (Parashar et al., 2009). The serogroup B and C can infect some mammal species such as humans, pigs, cattle, and dogs. Group E rotavirus is reported only in pigs. Whereas group G-F rotaviruses are reported only in poultry. Rotavirus is species specific. However, it has been continuously reported to cross-species transmission from animals to human via direct interspecies transmission or reassortment between strains (Martella et al., 2010).

Canine rotavirus (CRV) causes subclinical or mild gastroenteritis disease in puppy. Adult dogs had high prevalence of rotavirus (Rimmelzwaan et al., 1991).

Rotavirus strains G3P[3] and G3P[9] are predominant strains in dogs and cats in many countries including U.S.A and Europe (CU-1, A79-10, LSU79C-36, RS15, RV198/95, and RV52/96) (Mihalov-Kovacs et al., 2015). Although, CRV might be less-virulence in animals, but it is likely to cause a disease in human such as CRV strains Ro1845 and PA260/97. It has been reported that a new reassortment of canine/feline rotaviruses could be the causes of severe gastroenteritis outbreaks in human (Wu et al., 2012b). In Thailand, rotavirus strains G3P[3] and G3P[9], which are potential originated from cats and dogs, had been isolated from gastroenteritis children in Thailand (Theamboonlers et al., 2013a).

**The result of this dissertation was presented in 10 chapters including;**

1. Chapter I: introduction and literature reviews
2. Chapter II: Canine parainfluenza virus type 5 which have been published in the topic “Molecular detection and whole genome characterization of Canine Parainfluenza type 5 in Thailand” in Scientific Reports, 11, Article number: 3866 (2021).
3. Chapter III: Canine influenza virus which the manuscript is in preparation.
4. Chapter IV: Canine respiratory coronavirus which the manuscript is in preparation.
5. Chapter V: Canine Kobuvirus which have been published in the topic “First Detection and Genetic Characterization of Canine Kobuvirus in domestic dogs in Thailand” in BMC Veterinary Research; 15, Article number: 254 (2019)
6. Chapter VI: Norovirus which have been published in the topic “Human Norovirus Infection in Dogs, Thailand” in Emerging Infectious Disease; 2020;26(2):350-353.
7. Chapter VII: Canine parvovirus type 2 which have been published in the topic “Emergence of canine parvovirus type 2c in domestic dogs and cats from Thailand” in Transboundary and Emerging Disease; 2019 Jul;66(4):1518-1528.
8. Chapter VIII: Canine rotavirus which have been published in the topic “Molecular characterization identifies intra-host recombination and zoonotic

potential of canine rotavirus among dogs from Thailand” in *Transboundary and Emerging Disease*; 2020 Aug 9. doi: 10.1111/tbed.13778.

9. Chapter IX: Surveillance of potential respiratory and enteric zoonotic viruses in High-risk occupations in Bangkok, Thailand which the manuscript is in preparation.
10. Chapter X: Conclusions and recommendations.



## CHAPTER II

### CANINE PARAINFLUENZA TYPE 5

Parts of this work have been published in

#### Molecular detection and whole genome characterization of Canine Parainfluenza type 5 in Thailand

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#### 2.1 Abstract

Parainfluenza virus type 5 (PIV-5) causes respiratory infection in several animal species and humans. Canine parainfluenza virus type 5 (CPIV-5) causes respiratory disease in domestic dogs worldwide. In this study, we conducted a cross-sectional survey of CPIV-5 in dogs with respiratory symptoms from small animal hospitals in Thailand from November 2015 to December 2018. Our results showed that 32 out of 571 nasal swab samples (5.6%) were positive for CPIV-5 by RT-PCR specific to the NP gene. To characterize the viruses, three representative CPIV-5 were subjected to whole genome sequencing, and an additional ten CPIV-5 were subjected to HN, F, SH, and V/P gene sequencing. Pairwise sequence comparison and phylogenetic analysis showed that Thai CPIV-5 was closely related to the CPIV-5 isolated from China and Korea. In conclusion, this study constitutes a whole genome characterization of CPIV-5 from dogs in Thailand. The surveillance of CPIV-5 should be further investigated at a larger scale to determine the dynamics, distribution, and potential zoonotic transmission of CPIV-5.

**Keywords:** Characterization; Dogs; Parainfluenza type 5; Thailand

## 2.2 Introduction

Parainfluenza virus (PIV) is an enveloped, nonsegmented, single-stranded RNA virus. PIV-5 belongs to the family *Paramyxoviridae*, genus *Rubulavirus*. The virus consists of seven genes encoding 8 proteins (F, HN, SH, M, NP, V, P, and L) (Thomas et al., 1988). PIV can be classified into 5 types, designated PIV 1-5. PIV-1 to PIV-4 can cause upper and lower respiratory tract infections in humans, especially in infants and young children (Chew et al., 1998; Henrickson, 2003; Morgan et al., 2013; Ruampunpong et al., 2014). PIV-5 has been reported to infect and cause respiratory disease in several host species.

PIV-5 was first isolated in 1956 from rhesus and cynomolgus monkey kidney-cells (Hull et al., 1956). The virus was previously named simian virus type 5 (SV-5) according to the host of isolation. Then, SV-5 was renamed to PIV-5 and prefixed according to the isolated species (Chatziandreou et al., 2004). To date, the disease caused by PIV-5 in humans are still unclear. Some studies revealed that a virus serologically related to PIV-5 was associated with multiple sclerosis (MS), sclerosing panencephalitis (SSPE), Creutzfeldt-Jakob disease (CJD), pemphigus, atherosclerosis, Paget's disease, hepatitis and common cold in humans (Basle et al., 1985; Goswami et al., 1984; Goswami et al., 1987a). There were in vitro studies and need to be identified as such PIV-5 was found in human respiratory cells and might impact human respiratory diseases (Danjoh et al., 2009; Zhang et al., 2011).

PIV-5 has been reported in several host species including pigs, cattle, dogs, hamsters, ferrets, monkeys, calves, lesser pandas, and guinea pigs (Goswami et al., 1987a; Lee and Lee, 2013; Zhai et al., 2017). In pigs, PIV-5 co-infects with porcine reproductive and respiratory syndrome (PRRSV) and causes respiratory symptoms. In cattle, PIV-5 possibly causes severe respiratory illness and leads to a high morbidity rate in calves (Liu et al., 2015). In dogs, canine parainfluenza virus type 5 (CPIV-5) was first isolated from dogs with respiratory signs in 1967 and was first named canine parainfluenza virus type 2 (CPIV-2) due to it causing a respiratory disease similar to that of human parainfluenza type 2 (HPIV-2) (Binn et al., 1967). A subsequent study based on antigenic and sequence analyses revealed that CPIV-5 and HPIV-2 are different (Ellis and Krakowka, 2012). It has been reported that CPIV-5 is one of the

common pathogens of canine infectious respiratory disease (CIRD). CPIV-5 causes mild to moderate respiratory illness in dogs. Dogs can develop severe clinical signs if co-infected with other respiratory viruses or bacteria (Ajiki et al., 1982; Joffe et al., 2016; Viitanen et al., 2015). In some cases, CPIV-5 can cause neurological disorders especially in puppies including encephalitis, seizures, myoclonus, and posterior paresis (Baumgartner et al., 1982; Baumgartner et al., 1981). The cross-species transmission of CPIV-5 has been reported in coyotes, ferrets, and rodents (Davidson et al., 1992; Durchfeld et al., 1991).

Interspecies transmission of PIV-5 between canines and humans has not been reported. However, a study suggested that PIV-5 might be a potential zoonotic pathogen (Chen et al., 2012). Some studies have supported the hypothesis that genetic characteristics between PIV-5 isolated from canines and humans are highly similar with fewer nucleotide sequence variations (only 0.1% to 3% nucleotide difference) (Chatziandreou et al., 2004; Lazar et al., 1970; Randall et al., 1987). In addition, CPIV-5 can be grown in various cell lines especially human cell lines (2fTGH and HEP2) which might correlate with the host preference of the virus (Parisien et al., 2002). Since epidemiological and whole genome sequence information on CPIV-5 is still limited, in this study, we conducted a cross-sectional survey of CPIV-5 in dogs and characterized the whole genome of Thai CPIV-5.



## 2.3 Materials and Methods

### 2.3.1. Canine samples

From November 2015 to December 2018, a total of 571 nasal swab samples were collected from dogs with respiratory symptoms, including sneezing, nasal discharge, cough, and dyspnea. Sample collection was conducted at Chulalongkorn University's Veterinary Teaching Hospital and private small animal hospitals in Bangkok, Thailand. The animal demographic data, including age, sex, breed, contact history, and vaccination history, were recorded. This study was conducted under approval from the Institute of Animal Use and Care Committee (IACUC# 1731074), and all procedures were completed in accordance with the relevant guidelines and regulations.

### 2.3.2. Canine parainfluenza virus identification

RNA extraction from nasal swab samples was conducted by using the QIAmp viral RNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. Briefly, 140  $\mu$ l of nasal swab sample was lysed by Buffer AVL-carrier RNA and 560  $\mu$ l of ethanol. The mixture was centrifuged and transferred into a column, and then 500  $\mu$ l each of buffers AW1 and AW2 were added. Finally, the RNA was eluted by 50  $\mu$ l of buffer AVE. RNA was stored at  $-20^{\circ}\text{C}$  until use. CPiV-5 detection was performed by using a nested RT-PCR assay specific to the NP gene of PIV-5 (Table 2.1) (Posuwan et al., 2010b). Briefly, one-step nested RT-PCR was conducted in a total final volume of 25  $\mu$ l comprised of 3  $\mu$ l of template RNA, 12.5  $\mu$ l of 2x reaction mix, 0.6  $\mu$ l of 10  $\mu$ M forward (CPiV-F363) and reverse primer (CPiV-R538), 1.2  $\mu$ l of SuperScript III RT (Invitrogen, USA) and distilled water to a final volume of 25  $\mu$ l. The first round of PCR product was diluted 1:5 with distilled water and subjected to a second round by using the TopTaq Master Mix Kit (Qiagen, Germany). The final volume was 20  $\mu$ l, including 10  $\mu$ l of 2x TopTaq Master Mix, 1  $\mu$ l of 10  $\mu$ M forward (CPiV-F428) and reverse primer (CPiV-R538), 2  $\mu$ l of 10x coral load, and 1  $\mu$ l of DNA. For the first round of nested RT-PCR conditions, the reaction contained a cDNA synthesis step at  $55^{\circ}\text{C}$  for 30 minutes, an initial denaturation step

at 94°C for 2 min, 40 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s and extension at 68°C for 30s, and a final extension step at 68°C for 6 min. For the second round of nested PCR conditions, the reaction comprised an initial denaturation step at 94°C for 3 min, 35 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s and extension at 72°C for 30s and a final extension step at 72°C for 7 min. To confirm CPIV5, 4 µl of PCR product was run on a 1.5% agarose gel with red safe. The expected size of the positive CPIV-5 product was 188 bp. Statistical analysis by the Fisher's exact test was used to compare the proportion of CPIV-5 positivity among dogs categorized by the time of sample collection, age of dogs, and vaccination history.

### **2.3.3. Canine parainfluenza virus isolation**

To isolate CPIV-5, RT-PCR-positive nasal swabs were subjected to virus isolation by using a Vero cell monolayer (ATCC, USA) at the Faculty of Veterinary Science, Chulalongkorn University. In brief, nasal swab sample were filtered with a 0.22 µm filter and inoculated onto a Vero cell monolayer containing Dulbecco's minimal essential medium (DMEM, Gibco), 2% fetal bovine serum (FBS, Gibco), and gentamycin sulfate (50 µg/ml) at 37°C in 5% CO<sub>2</sub>. If a cytopathic effect (CPE) was observed, the virus was harvested by centrifugation at 1,000 rpm for 10 min. The cell suspension was then screened for CPIV-5 by using nested RT-PCR as previously described (Liu et al., 2017; Posuwan et al., 2010b). The isolated viruses were kept at -80°C for the pathogenesis studies in the future.

### **2.3.4. Canine parainfluenza virus characterization**

In this study, Thai-CPIV-5 was selected for either whole genome sequencing (n=3) or F, HN, V/P, and SH gene sequencing (n=10). The representative CPIV-5 was selected based on epidemiological and demographic data such as the age of the dog, date of isolation, breed, and vaccination history. For whole genome sequencing, nucleotide sequences of each virus gene were amplified by PCR using oligonucleotide primers specific to each gene. The primers were synthesized per previous report and newly designed by using Primer 3 plus (Table 2.1) (Liu et al.,

2017; Rozen and Skaletsky, 2000). Nucleotide sequencing was conducted at the 1<sup>st</sup> Base Laboratories Sdn Bhd, Malaysia. The nucleotide sequences were validated and assembled by SeqMan software v.5 v.5.03 (DNASTAR Inc., Wisconsin, USA). In this study, nucleotide sequences of Thai CPIV-5 were submitted to the GenBank database under the accession numbers MT603999-MT604041 (Table 2.1).

Phylogenetic and genetic analyses were carried out by comparing nucleotide sequences of Thai CPIV-5 with those of PIV-5 available from the GenBank database. The reference nucleotide sequences of PIV-5 were retrieved based on geographic location, and host species including human PIV-1 (KF530221), swine PIV-1 (S033N; JX857410), human PIV-2 (NC003443), human PIV-3 (NC001796), swine PIV-3 (Texas-81; EU439429), and human PIV-4 (KF483663). Reference PIV-5 includes human strains (AGS; KX060176, DEN; JQ743322, MIL; JQ743326, MEL; JQ743325, RQ; JQ743327, LN; JQ743324), a rhesus macaque kidney cell strain (W3A; JQ743318.1), canine strains (HeN0718; KY114804, CC-14; KP893891, H221; JQ743323, 78524; JQ743319, CPI+; JQ743321, CPI-; JQ743320, 08-1990; KC237063, D277; KC237065, 1168-1; KC237064), swine strains (SER; JQ743328, KNU-11; KC852177), a cattle strain (PV5-BC14; KM067467), a lesser panda strain (ZJQ-221; KX100034) and a pangolin strain (CAN; MH362816). Phylogenetic analysis of CPIV-5 was performed by using MEGA v.7.0 (Tempe, AZ, USA) with the neighbor-joining method with the Kimura 2-parameter with 1,000 bootstrap replicates (Tamura et al., 2013). For genetic analysis, the nucleotide sequences and deduced amino acids of CPIV-5 were aligned and compared using MegAlign software v.5.03 (DNASTAR Inc., Wisconsin, USA).

### **2.3.5. Statistical analysis**

Categorical data corresponding to the time of sample collection, age of dogs, and vaccination history were analyzed using the Fisher's exact test (<https://www.socscistatistics.com/tests/fisher>). A p-value of <0.05 was considered as statistically significant.

**Table 2.1.** Nucleotide sequences of primers used for CPIV-5 detection and sequencing in this study.

Primer name	Forward (5'-3')	Primer name	Reward (5'-3')	Position *	Gene	Product size	Reference
<b>Primer detection</b>							
CPIV- F363	GGGTAGAGATCGATGGCTTTGA	CPIV-R538	GCGCAGTCATGCACCTTGC AAGT		NP	188	Posuwan, N. et al, 2010
CPIV-F428	GCCGTGGAGAGATCAATGCCTAT						
<b>Primer sequencing</b>							
PIV5_ 1F	AGGGAAAATGAAGTGGTGA	PIV5_ 1R	AGCCTATTCCTATAGCATAGCT	5-1173	NP	1168	This study
PIV5_ 2F	GGCTCGAGGAATATCCAGA	PIV5_ 2R	GCGGCATTC AAGTCATCTGT	813-1679	NP	866	This study
PIV5_ 3F	GGTCTAACTCAAGCCGAACG	PIV5_ 3R	TCGGGGTGGAGTCCAGACC	1324-2194	NP	870	This study
PIV5_ 4F	CGGACGGGTTAGTAACAAGC	PIV5_ 4R	TCCCTGCTTTGAGTCCAACT	1814-2657	V/P	843	This study
PIV5_ 5F	CAGTTCCTCCAAACCGATTTTA	PIV5_ 5R	TGCAAAAATGTCGGAATGAA	2334-3683	V/P	1349	This study
PIV5_ 6F	CGACC AACTGCAACAAGTG	PIV5_ 6R	TCTCACAGAGAAGAGAGATGC	2835-4176	M	1341	This study
PIV5_ 7F	CCACTGGTGACGTTCTGTAAA	PIV5_ 7R	AAGTTCCCGACATTTGTTG	3311-4663	M	1352	This study
PIV5_ 8F	AGGTCACATCCCTGAC AAG	PIV5_ 8R	TGCGGGGTTTGTAAATTTGAT	3816-5194	F	1378	This study
PIV5_ 9F	TCGAGATTACACACCTGAAA	PIV5_ 9R	TCCATCAAAACAGACGAATC	4307-5671	F	1364	This study
PIV5_ 10F	GTTGATTC AACTCGGAGGA	PIV5_ 10R	TGAGATCAGACCGTGTAGTGG	4828-6167	F	1339	This study
PIV5_ 11F	GGCCAGATTGTGGGATTAGA	PIV5_ 11R	TCGAAATAATACTGGCAAAGTG	5318-6656	F, SH	1338	This study
PIV5_ 12F	AACCTACAA TAGCACCATCAAGC	PIV5_ 12R	ACATGATCTCTGGCATCCATT	5836-7169	HN	1333	This study
PIV5_ 13F	CATTGTCGTGCTTAATCGAA	PIV5_ 13R	TTCTGGAGAGCGGTAGAAA	6338-7679	HN	1341	This study
PIV5_ 14F	CTCTGCAGTCGCTTACCTCT	PIV5_ 14R	CTTGACCGCTTGATCCAAAT	6838-8161	HN	1323	This study
PIV5_ 15F	TCAACAGAGAGGGATGACTAC	PIV5_ 15R	GCCAATGGCCCTTTCTAAG	7338-8679	HN	1341	This study
PIV5_ 16F	TGGCCTATGACCATGCTGTA	PIV5_ 16R	AAAATGTTGTGACGACCTTCG	7831-9189	L	1358	This study
PIV5_ 17F	CAGGTC TGACCCAGCAAATC	PIV5_ 17R	GGCCATACTCCATGATGCTT	8315-9689	L	1374	This study
PIV5_ 18F	AATGCGGGTACTTCCCAAGT	PIV5_ 18R	CCAGCATGGTTCGCTAAAAG	8809-10187	L	1378	This study
PIV5_ 19F	ATGTCAGATCCCAATCC CAGA	PIV5_ 19R	AGCCCTTCAATTC AACTCT	9340-10658	L	1318	This study
PIV5_ 20F	CGCAGGTGAGGAACTCAGTA	PIV5_ 20R	TCCAGGAAITTGACACTTGAGG	9828-11173	L	1345	This study
PIV5_ 21F	AATCACGTC ACTCCAAGCAA	PIV5_ 21R	TCTGTTTTCTCTCCCGACA	10343-11656	L	1313	This study
PIV5_ 22F	TTGAAAAAGACTATTGCTTTTAGA	PIV5_ 22R	TACACTCTGCCAGCTTAAGA	10808-12178	L	1370	This study
PIV5_ 23F	TTCCGCAGTTGCAGATCTTA	PIV5_ 23R	GCGGGTCTTCATCAAAATACAA	11307-12656	L	1349	This study
PIV5_ 24F	AGCATCGACATTTGCAAGGAG	PIV5_ 24R	TCCATTGGAGAGACATTTGA	11842-13194	L	1352	This study
PIV5_ 25F	GGATGATGGCACAACTACCC	PIV5_ 25R	CTCCAAGTTTGCATTGGACTC	12315-13677	L	1362	This study
PIV5_ 26F	TGCCATTGTTGCATCAGACT	PIV5_ 26R	AAGCTTGCACTTGACTCCAAA	12825-14187	L	1362	This study
PIV5_ 27F	AAGGGGTTCTCTCTGATGA	PIV5_ 27R	AAGTTGCGACTGGCTCGATA	13324-14674	L	1350	This study
PIV5_ 28F	GCTACCAGAGGTGATCAITTT	PIV5_ 28R	TATTTAGATTTCTCCGCATCG	13828-15206	L	1378	This study

\* Position based on CPIV-5 strain 08-1990 (KC237063)

## 2.4 Results

In this study, we investigated canine parainfluenza type 5 (CPIV-5) infection in dogs with respiratory symptoms from November 2015 to December 2018. Our results showed that 5.6% (32/571) of nasal swab samples were positive for CPIV-5. From 3 years of surveillance, the highest occurrence of CPIV-5 was observed in November 2016 (41.7%), followed by December 2016 (33.3%) with statistical significance  $p < 0.05$  when compared to other years (Table 2.2). Regarding the relationship between CPIV-5 infection and age group, the occurrences of CPIV-5 was statistically more frequent in dogs  $< 1$  year (10.0%; 24/240) than in dogs older than 5 years (3.3%; 4/120) and dogs 1-4 years (1.9%; 4/211) ( $p = 0.0349$  and  $0.0003$ ,  $p < 0.05$ , respectively). Regarding the relationship between CPIV-5 infection and vaccination history, the occurrence of CPIV-5 infection in dogs with incomplete CPIV-5 vaccination (10.4%; 28/269) was higher than in dogs fully vaccinated (1.3%; 4/302), with statistical significance ( $p < 0.05$ ).

**Table 2.2.** Details of the sample collection and detection of CPIV- 5

Year	Month	Total samples	Number positive for CPIV5 (%)
2015	Nov	7	0
	Dec	10	0
2016	Jan	9	1 (11.1%)
	Feb	11	1 (9.1%)
	Mar	7	0
	Apr	13	1 (7.7%)
	May	12	1 (8.3%)
	June	3	0
	July	15	0
	Aug	10	0
	Sep	9	0
	Oct	11	1 (9.1%)
	Nov	12	5 (41.7%)*
	Dec	15	5 (33.3%)*
2017	Jan	12	3 (25.0%)*
	Feb	26	0
	Mar	32	5 (15.6%)
	Apr	24	0
	May	11	0
	June	11	0
	July	19	0
	Aug	17	0
	Sep	23	1 (4.4%)
	Oct	21	0
	Nov	13	0
	Dec	33	3 (9.1%)
2018	Jan	19	1 (5.3%)
	Feb	17	2 (11.8%)
	Mar	14	0
	Apr	18	0
	May	9	1 (11.1%)
	June	19	0
	July	21	0
	Aug	18	0
	Sep	11	1 (9.1%)
	Oct	10	0
	Nov	15	0
	Dec	14	0
Total		571	32 5.60%)38

\* Statistical significance when compared to the same month of other years

#### 2.4.1. Genetic characteristics of Thai canine parainfluenza type 5

In this study, we selected and characterized representatives of Thai CPIV-5 for whole genome sequencing (n=3; CU-D133, CU-D151 and CU-D20804) and F, HN, V/P and SH gene sequencing (n=10) (Table 2.3). Our results showed that the genome size of Thai CPIV-5 is 15,207 bp, containing seven genes as 3'-N-V/P-M-F-SH-HN-L-5'. Whole genome sequence analysis showed that Thai CPIV-5 possessed high nucleotide identity to the reference PIV5 with 96.1-99.4% nucleotide identities but low percentages of nucleotide identities with PIV-1 to PIV-4 (44.5- 63.1% nucleotide identities). Comparing PIV-5, the whole genome of Thai CPIV-5 was closely related to Chinese CPIV-5 (HeN0718, 99.2% nucleotide identities) and Korean CPIV-5 (D277 and 08-1990, 99.4% and 99.2% nucleotide identities) (Table 2.4). For phylogenetic analysis, Thai CPIV-5 (n=3) was grouped with PIV-5 from humans, pigs, dogs, lesser panda, and pangolins but separated from clusters of PIV-1 to PIV-4. The phylogenetic tree of the whole genome of PIV-5 could be divided into subgroups, e.g., human, and simian subgroup, cattle and swine subgroup and canine subgroup. Thai CPIV-5 was grouped in the canine subgroup with CPIV-5 from China (HeN0718) and Korea (D277 and 08-1990) (Figure 2.1).

Pairwise comparison of nucleotide sequences showed that the HN, F, V/P and SH genes of Thai CPIV-5 possessed high nucleotide identities to Chinese CPIV-5 (HeN0718; 96.9-99.5%) and Korean CPIV-5 (D277 and 08-1990; 99.3-99.9%), which were similar to the whole genome sequences (Table 2.4). The phylogenetic analysis of the F, HN, and V/P genes showed that Thai CPIV-5 was grouped with Chinese CPIV-5 (HeN0718) and Korea CPIV-5 (D277 and 08-1990) (Figure 2.2). Moreover, the M, NP and L genes of Thai CPIV-5 (CU-D131, CU-D151 and CU-D20804) had the highest nucleotide identities to Korean CPIV-5 (D277; 99.6%-99.9%). The phylogenetic analysis results showed that the M, NP and L genes were also closely related to CPIV-5 from Chinese and Korean strains (Figure 2.2).

Genetic analysis of the HN gene (1698 nucleotides, 565 amino acids) of Thai CPIV-5 showed that amino acid residues at the receptor binding site (positions 186-190) and cleavage site (positions 390 and 523) of the HN protein contained QDHVS (186-190), E390 and Y523. Amino acid residues at the stalk regions contained S60,

Y77, L90, E91 and Q102 identical to the reference PIV-5. Amino acid residues at positions 37, 342, 437, and 457, which correlated with neutralizing antibodies, contained E37, K342, T437, and F457. It is noted that Q342K was only observed in all Thai CPIV-5, which was identical to CPIV-5 from China (HeN0718) and Korea (D277 and 08-1990) but not in other CPIV-5 and human PIV-5 (Table 2.5). Amino acid residues related to host preference (human specific) at I22L, A49S, R57G, T254A, N318S, K460T and M536T were analyzed. Thai CPIV-5 contained I22, A49, R57, T254, N318, K460 and M536, which are not human specific amino acids. Unique amino acids for Thai, Chinese and Korean CPIV-5 were also observed at T19I, K43E, T62I, T141A, F252L, F353L and G446R suggesting unique subclustered characteristics (Table 2.6).

Genetic analysis of the F gene showed a low level of genetic variation. Amino acid residues related to host preference (human specific) were observed at T3I, S19G, I301M, T438S, L498F, S530Q and R536Q. One Thai CPIV-5 (CU-D151) contained R536Q similar to some human PIV-5 (DEN, MIL, RQ, and LN). Moreover, Thai CPIV-5 contained 22P and 443P, which were similar to PIV-5 from humans and pigs suggesting potential human preference characteristics (Bose et al., 2013; Ito et al., 2009; Rima et al., 2014) (Table 2.7). Genetic analysis of the V/P gene showed that amino acid related to viral RNA synthesis contained S157, T286 and K254 similar to most CPIV-5 (Table 2.8)

Genetic analysis of the SH gene showed that Thai CPIV-5 (CU-D58, CU-D103, CU-D133, CU-D151, CU-D376, CU-D381, CU-D406, and CU-D20804) contained a non-synonymous substitution at the start codon (M1T). Distinct nucleotide substitutions at T133C were observed and resulted in the extension of four amino acids at the stop codon, similar to those of CPIV-5 from China and Korea. Thus, the SH protein of Thai, Chinese, and Korean CPIV-5 is four amino acids longer than that of the reference PIV-5 (Table 2.8 and Figure 2.3).



**Table 2.3.** Description of canine parainfluenza type 5 (CPIV-5) characterized in this study.

Virus	Collection date	Age	Breed	Vaccination history	CPIV-5 detection	Sequencing	# GenBank
CU-D58	Jan 16	3 mts	Siberian Husky	I	+	F, HN, SH, V/P *	MT604002-05
CU-D103	Feb 16	2 mts	Bully	I	+	F, HN, SH, V/P	MT604006-09
CU-D133	Apr 16	> 7 yrs	Golden retriever	C	+	WGS**	MT603999
CU-D151	May 16	3 mts	Pomeranian	I	+	WGS	MT604000
CU-D373	Nov 16	3 mts	Pomeranian	I	+	F, HN, SH, V/P	MT604011-13
CU-D376	Dec 16	> 1 yrs	Mixed	I	+	F, HN, SH, V/P	MT604014-17
CU-D381	Dec 16	3 mts	Pekingese	I	+	F, HN, SH, V/P	MT604018-21
CU-D399	Jan 17	4 mts	Pomeranian	I	+	F, HN, SH, V/P	MT604022-25
CU-D400	Jan 17	7 mts	Pomeranian	I	+	F, HN, SH, V/P	MT604026-29
CU-D406	Jan 17	3 mts	Pomeranian	I	+	F, HN, SH, V/P	MT604030-33
CU-D466	Mar 17	2 mts	Mixed	I	+	F, HN, SH, V/P	MT604034-37
CU-D585	Sep 17	3 mts	Mixed	I	+	F, HN, SH, V/P	MT604038-41
CU-D20804	Feb 18	4 mts	Mixed	I	+	WGS	MT604001
CU-D361	Oct 16	2 mts	Pomeranian	I	+	-	-
CU-D369	Nov 16	6 mts	Pomeranian	I	+	-	-
CU-D370	Nov 16	10 yrs	Shih-Tzu	C	+	-	-
CU-D371	Nov 16	12 yrs	Poodle	C	+	-	-
CU-D372	Nov 16	4 mts	Pomeranian	I	+	-	-
CU-D377	Dec 16	> 1 yrs	Mixed	I	+	-	-
CU-D380	Dec 16	10 mts	Mixed	I	+	-	-
CU-D390	Dec 16	> 5 yrs	Mixed	C	+	-	-
CU-D483	Mar 17	>1 yrs	Mixed	I	+	-	-
CU-D489	Mar 17	>1 yrs	Mixed	I	+	-	-
CU-D493	Mar 17	6 mts	Mixed	I	+	-	-
CU-D497	Mar 17	6 mts	Mixed	I	+	-	-
CU-D20273	Dec 17	2 mts	Pomeranian	I	+	-	-
CU-D20277	Dec 17	2 mts	Bully	I	+	-	-
CU-D20364	Dec 17	3 mts	Pomeranian	I	+	-	-
CU-D20384	Jan 18	3 mts	Mixed	I	+	-	-
CU-D20803	Feb 18	3 mts	Mixed	I	+	-	-
CU-D21496	May 18	2 mts	Mixed	I	+	-	-
CU-D22309	Sep18	3 mts	Samoyed	I	+	-	-

C; Complete vaccination

**Table 2.4.** Pairwise comparison of whole genome nucleotide sequences of Thai CPiV-5 (CU-D151) with reference parainfluenza viruses

Virus	Accession No.	Host	Location	(%) Nucleotide identity										
				WGS	N (1530 nt)	F (1590-1656 nt)	HN (1698 nt)	SH (135 nt)	V (669 nt)	P (1177 nt)	M (1134 nt)	L (6768 nt)		
CU-D151	This study	Canine	Thailand	100.0	100.0	100.0	100.0	(-)	100.0	100.0	100.0	100.0	100.0	100.0
CU-D133	This study	Canine	Thailand	99.1	99.3	99.0	99.5	(-)	99.0	98.9	99.4	99.3	99.3	99.3
CU-D20804	This study	Canine	Thailand	99.2	99.5	99.4	98.8	(-)	99.3	99.2	99.1	99.2	99.2	99.2
CU-D58	This study	Canine	Thailand	(-)	99.5	99.5	99.2	(-)	99.3	99.2	(-)	(-)	(-)	(-)
CU-D103	This study	Canine	Thailand	(-)	99.3	99.0	99.6	(-)	98.8	98.8	(-)	(-)	(-)	(-)
CU-D373	This study	Canine	Thailand	(-)	(-)	99.2	97.2	(-)	99.0	99.1	(-)	(-)	(-)	(-)
CU-D376	This study	Canine	Thailand	(-)	(-)	97.3	99.9	(-)	99.3	99.2	(-)	(-)	(-)	(-)
CU-D381	This study	Canine	Thailand	(-)	(-)	99.5	99.8	(-)	99.3	99.2	(-)	(-)	(-)	(-)
CU-D399	This study	Canine	Thailand	(-)	(-)	96.8	98.8	(-)	97.2	97.0	(-)	(-)	(-)	(-)
CU-D400	This study	Canine	Thailand	(-)	(-)	99.1	99.2	(-)	99.0	99.1	(-)	(-)	(-)	(-)
CU-D406	This study	Canine	Thailand	(-)	(-)	99.5	99.4	(-)	99.3	99.2	(-)	(-)	(-)	(-)
CU-D466	This study	Canine	Thailand	(-)	(-)	99.3	99.5	(-)	99.1	99.2	(-)	(-)	(-)	(-)
CU-D585	This study	Canine	Thailand	(-)	(-)	99.3	100.0	(-)	99.1	99.2	(-)	(-)	(-)	(-)
<b>Reference PIV-5</b>														
AGS	KX060176	AGS cell	USA	96.1	96.2	95.0	95.8	(-)	95.7	95.9	95.6	96.7	96.7	96.7
DEN	JQ743322	Human	UK	96.6	96.6	95.6	96.4	(-)	96.1	96.1	96.0	97.0	97.0	97.0
MIL	JQ743326	Human	UK	96.5	96.5	95.6	96.3	(-)	96.0	96.0	96.0	97.0	97.0	97.0



**Table 2.5.** Genetic analysis of the HN gene of Thai CPIV-5 and reference PIV-5 at the receptor binding, cleavage site and stalk region

Virus	Host	HN gene											
		HN gene				Receptor binding site	Cleavage site		HN stalk				
		37	342	437	457	186-190	390	523	60	77	90	91	102
<b>Reference PIV-5</b>													
AGS	AGS Cell	E	K	T	A	QDHVS	E	Y	S	Y	L	E	H
W3A	Macaque cell	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
DEN	Human	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
MIL	Human	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
MEL	Human	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
RQ	Human	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
LN	Human	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
SER	Swine	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
KNU-11	Swine	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
PV5-BC14	Calve	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
ZJQ-221	Lesser panda	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
CAN	Pangolin	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
H221	Canine	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
78524	Canine	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
CPI+	Canine	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
CPI-	Canine	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
08-1990	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
D277	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
1168-1	Canine	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
CC-14	Canine	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
HeN0718	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
<b>This study</b>													
CU-D58	Canine	E	K	I	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D103	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D133	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D151	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D373	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D376	Canine	E	K	I	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D381	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D399	Canine	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D400	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D406	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D466	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D585	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D20804	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q

**Table 2.6.** Genetic analysis of the HN gene of Thai CPIV-5 and reference PIV-5 at the human-specific residues

Virus	Host	Location	Primate specific amino acid						Lineage specific amino acid*							
			22	49	57	254	318	460	536	19	43	62	141	252	353	446
<b>Reference PIV-5</b>																
AGS	AGS Cell		L	S	G	A	S	T	T	T	K	T	T	F	F	G
DEN	Human	UK	L	S	G	A	S	T	T	T	K	T	T	F	F	G
MIL	Human	UK	L	S	G	A	S	T	T	T	K	T	T	F	F	G
MEL	Human	UK	L	S	G	A	S	T	T	T	K	T	T	F	F	G
RQ	Human	UK	L	S	G	A	S	T	T	T	K	T	T	F	F	G
LN	Human	UK	L	S	G	A	S	T	T	T	K	T	T	F	F	G
W3A	Macaque cell		I	A	R	A	N	T	M	T	K	T	T	F	F	G
SER	Swine	Germany	I	A	R	T	N	K	M	T	K	T	T	F	F	G
KNU-11	Swine	South Korea	I	A	R	T	N	K	M	T	K	T	T	F	F	G
PV5-BC14	Calve	China	I	A	R	T	N	K	M	T	K	T	T	F	F	G
ZJQ-221	Lesser panda	China	I	A	R	T	N	K	M	T	K	T	T	F	F	G
CAN	Pangolin	China	I	A	R	T	N	K	M	T	K	T	T	F	F	G
H221	Canine	UK	I	A	R	T	N	K	I	T	K	T	T	F	F	G
78524	Canine	UK	I	A	R	T	N	K	M	T	K	T	T	F	F	G
CPI+	Canine	USA	I	A	R	T	N	K	M	T	K	T	T	L	F	G
CPI-	Canine	USA	I	A	R	T	N	K	M	T	K	T	T	L	F	G
08-1990	Canine	South Korea	I	A	R	T	N	K	M	I	E	I	A	L	L	R
D277	Canine	South Korea	I	A	R	T	N	K	M	I	E	I	A	L	L	R
1168-1	Canine	South Korea	I	A	R	T	N	K	M	T	K	T	T	F	F	G
CC-14	Canine	China	I	A	R	T	N	K	I	T	K	T	T	F	F	G
HeN0718	Canine	China	I	A	R	T	N	K	M	I	E	I	A	L	L	R
<b>This study</b>																
CU-D58	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D103	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D133	Canine	Thailand	I	A	R	T	N	K	I	I	E	I	A	L	L	R
CU-D151	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D373	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D376	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D381	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D399	Canine	Thailand	I	A	R	T	N	K	M	T	K	T	I	F	F	G
CU-D400	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D406	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D466	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D585	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D20804	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R

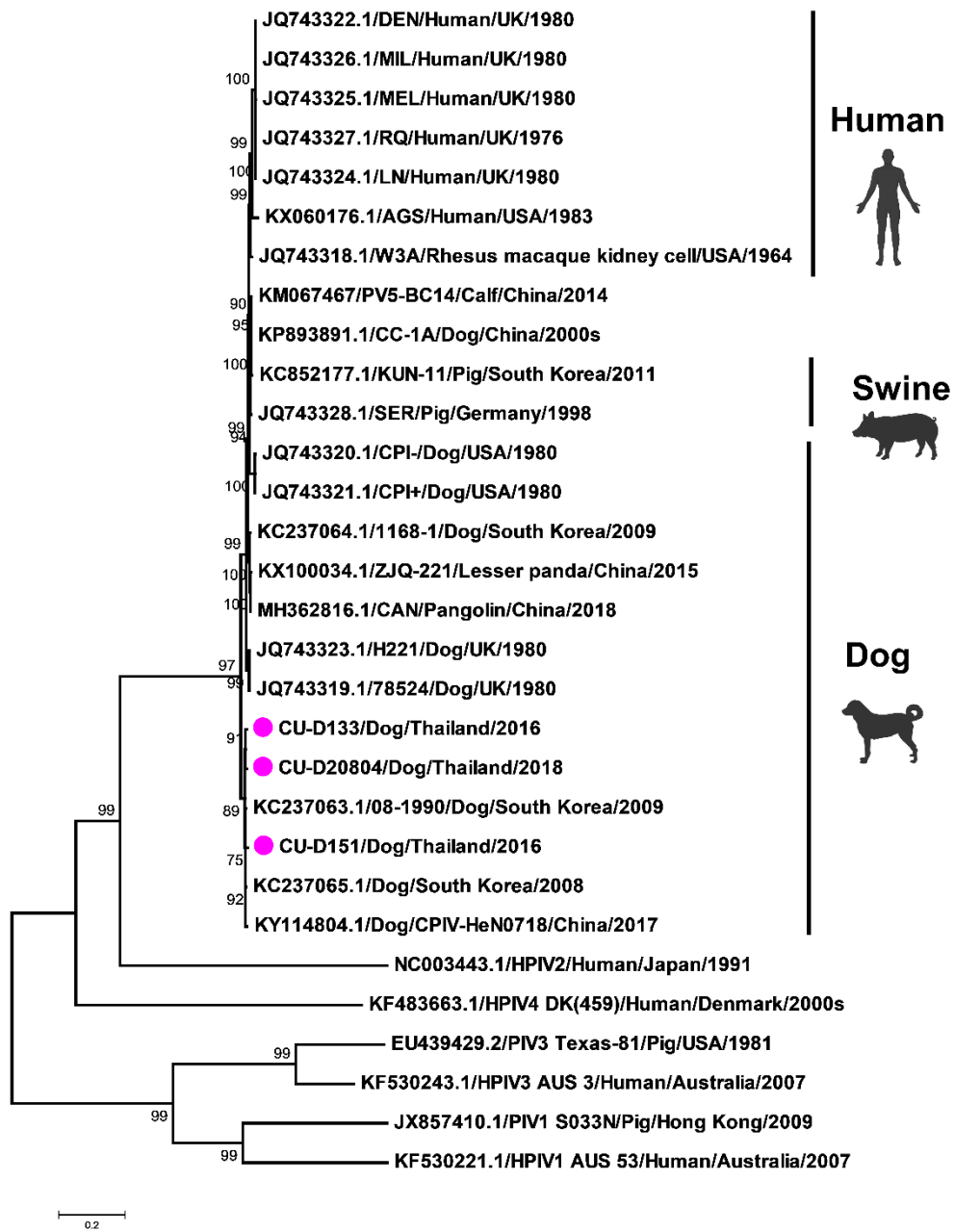
\* Lineage: CPIV-5 sublineage; Thai, Chinese, and Korean sublineages

**Table 2.7.** Genetic analysis of the F gene of Thai CPIV-5 and reference PIV

Virus	Host	Primate specific amino acid								
		L22P	S443P	3	19	310	438	498	530	536
<b>Reference PIV-5</b>										
DEN	Human	P	P	I	G	M	S	F	Q	Q
MIL	Human	P	P	I	G	M	S	F	Q	Q
MEL	Human	P	P	I	G	M	S	F	-	-
RQ	Human	P	P	I	G	M	S	F	Q	Q
LN	Human	P	P	I	G	M	S	F	Q	Q
AGS	Human	P	P	T	S	M	S	L	Q	Q
W3A	Macaque cell	P	S	T	S	M	S	L	-	-
SER	Swine	P	P	T	S	I	T	L	S	R
KNU-11	Swine	L	P	T	S	I	T	L	S	R
HLJ2015_DP1-1	Swine	P	P	T	S	I	T	L	S	R
PV5-BC14	Calve	L	P	T	S	I	T	L	S	R
ZJQ-221	Lesser panda	P	P	T	S	I	T	L	S	R
CC-14	Canine	L	P	T	S	I	T	L	S	R
H221	Canine	P	P	T	S	I	T	L	S	R
78524	Canine	P	P	T	S	I	T	L	S	R
CPI+	Canine	P	P	T	S	I	T	L	S	R
CPI-	Canine	P	P	T	S	I	T	L	S	R
08-1990	Canine	P	P	T	S	I	T	L	S	R
D277	Canine	P	P	T	S	I	T	L	S	R
1168-1	Canine	P	P	T	S	I	T	L	S	R
HeN0718	Canine	P	P	T	S	I	T	L	S	R
<b>This study</b>										
CU-D58	Canine	P	P	T	S	I	T	L	S	R
CU-D103	Canine	P	P	T	S	I	T	L	S	R
CU-D133	Canine	P	P	T	S	I	T	L	S	R
CU-D151	Canine	P	P	T	S	I	T	L	S	Q
CU-D373	Canine	P	P	T	S	I	T	L	S	R
CU-D376	Canine	P	P	T	S	I	T	L	S	R
CU-D381	Canine	P	P	T	S	I	T	L	S	R
CU-D399	Canine	P	P	T	S	I	T	L	S	R
CU-D400	Canine	P	P	T	S	I	T	L	S	R
CU-D406	Canine	P	P	T	S	I	T	L	S	R
CU-D466	Canine	P	P	T	S	I	T	L	S	R
CU-D585	Canine	P	P	T	S	I	T	L	S	R
CU-D20804	Canine	P	P	T	S	I	T	L	S	R

**Table 2.8.** Genetic analysis of the V/P, SH and non-coding region of Thai CPIV-5 and reference PIV-5.

Virus	Host	V/P					SH	Insertion at position 6506-6511 (6-nt)	Insertion at position 8394 (1-nt)
		S157F	T286A	K254R	T293K	S308A	Start codon		
<b>Reference PIV-5</b>									
AGS	AGS Cell	S	T	K	T	S	M	No	A
DEN	Human	F	T	K	T	S	M	No	No
MIL	Human	F	T	K	T	S	M	No	No
MEL	Human	F	T	K	T	S	M	No	No
RQ	Human	F	T	K	T	S	M	AAAGAA	No
LN	Human	F	T	K	T	S	M	AAAGAA	No
W3A	Macaque cell	S	T	K	T	S	M	No	No
SER	Swine	S	T	K	K	S	M	No	No
KNU-11	Swine	S	T	K	K	S	T	No	No
PV5-BC14	Calve	S	T	K	K	S	T	No	No
ZJQ-221	Lesser panda	S	T	K	K	S	T	No	No
CC-14	Canine	S	T	K	K	S	T	No	No
H221	Canine	S	T	K	K	S	M	No	No
78524	Canine	F	T	K	K	S	M	No	No
CPI+	Canine	F	T	K	K	S	T	No	No
CPI-	Canine	F	T	K	K	S	T	No	No
08-1990	Canine	S	T	K	K	S	M	No	No
D277	Canine	S	T	K	K	S	M	No	No
1168-1	Canine	S	T	K	K	S	M	No	No
<b>This study</b>									
CU-D58	Canine	S	T	K	K	S	T	N/A	N/A
CU-D103	Canine	S	T	K	K	S	T	N/A	N/A
CU-D133	Canine	S	T	K	K	S	T	No	No
CU-D151	Canine	S	T	K	K	S	T	No	No
CU-D373	Canine	S	T	K	K	S	M	N/A	N/A
CU-D376	Canine	S	T	K	K	S	T	N/A	N/A
CU-D381	Canine	S	T	K	K	S	T	N/A	N/A
CU-D399	Canine	S	T	K	K	S	M	N/A	N/A
CU-D400	Canine	S	T	K	K	S	M	N/A	N/A
CU-D406	Canine	S	T	K	K	S	T	N/A	N/A
CU-D466	Canine	S	T	K	K	S	M	N/A	N/A
CU-D585	Canine	S	T	K	K	S	M	N/A	N/A
CUD20804	Canine	S	T	K	K	S	T	No	No

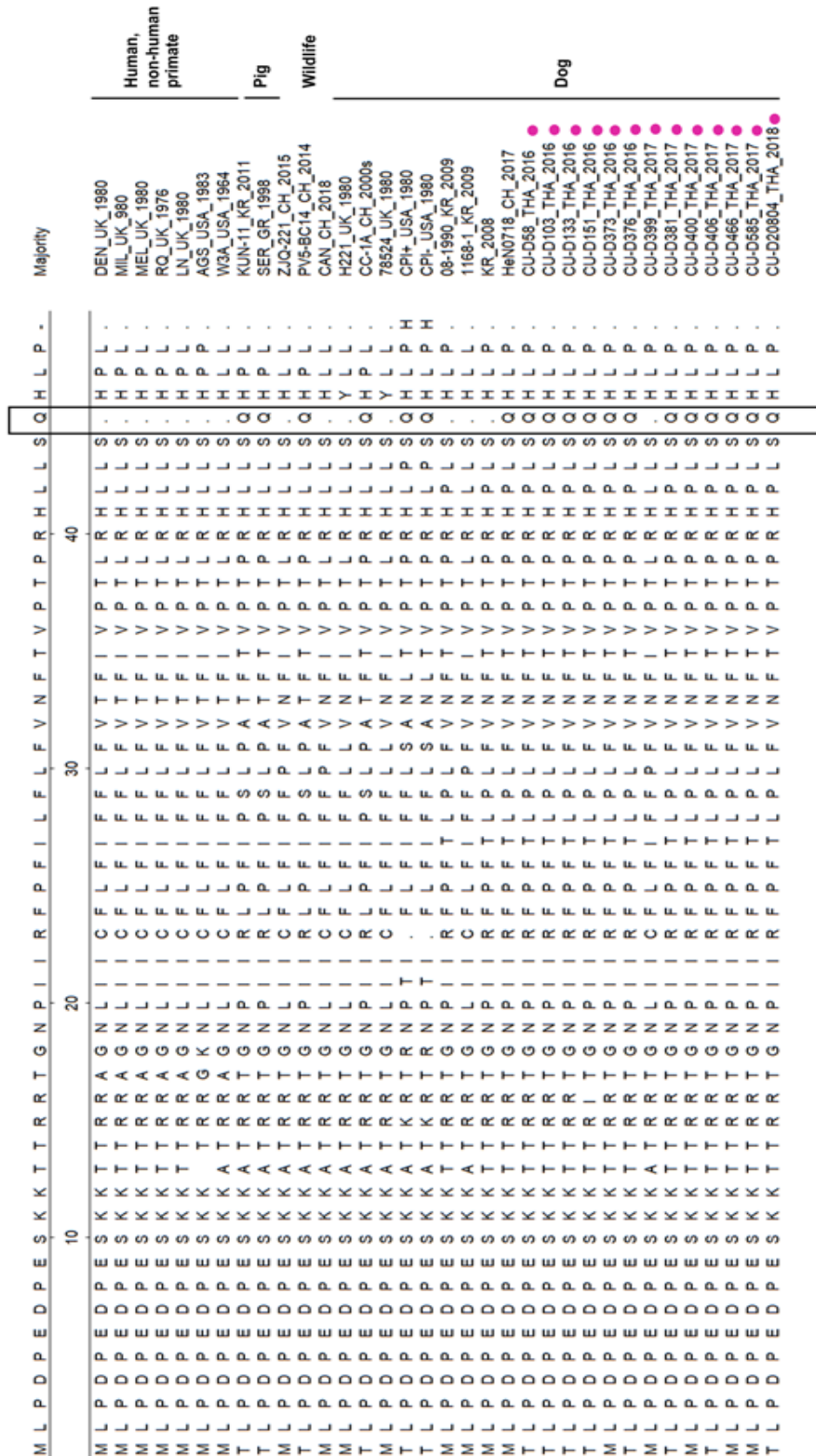


**Figure 2.1.** Phylogenetic tree of whole genome of Thai CPIV-5 and reference PIV1-5. Pink circles indicate Thai CPIV-5 in this study.





Figure 2.2. Phylogenetic trees of the HN, F, M, V/P, NP, and L genes of Thai CPV-5 and reference PIV-1-5. Pink circles indicate Thai CPV-5 in this study.



**Figure 2.3.** Alignment of deduced amino acids of SH gene of Thai CPIV-5 and reference PIV-5. The box indicates amino acid substitution at the stop codon (Q). Pink circles indicate Thai CPIV-5 in this study.

## 2.5 Discussion

Parainfluenza virus type 5 (PIV-5) can infect and cause respiratory diseases in various mammals. Canine parainfluenza virus type 5 (CPIV-5) is highly contagious and causes mild to moderate respiratory diseases in dogs worldwide. Coinfection with CPIV-5 and other viruses or bacteria can cause more virulent clinical signs. This study revealed the occurrence of CPIV-5, which was relatively high during the winter season in Thailand (November to January). A similar finding of high occurrence detected in the cold season has also been reported (Monteiro et al., 2016). CPIV-5 could be detected in younger dogs (< 1 year) more than in older dogs. Dogs of all ages could be infected with CPIV-5, but younger dogs (<1 year) are more susceptible. This observation is in agreement with a previous report that CPIV-5 could be observed more in younger dogs than in dogs in other age groups (Ellis et al., 2011; Mochizuki et al., 2008; Seyfiabad Shapouri et al., 2016). Regarding vaccination history, CPIV-5 infection was higher in dogs with incomplete vaccination (10.4%) than in dogs with complete vaccination (1.32%). The CPIV-5 vaccine used in Thailand was modified live CPIV-5 combined with other pathogens (e.g., canine distemper virus, canine parvovirus, and canine coronavirus). Some studies have suggested that vaccinated dogs can show mild clinical signs and shed the virus after infection (Emery et al., 1976). It is noted that, the CPIV-5 characterized in this study was obtained from nasal swabs of dogs with and without vaccination. A previous study revealed that whole genome sequences of CPIV-5 vaccine was identical with PIV-5 strain W3A, which different from Thai-CPIV-5 (Erles et al., 2004). Moreover, all three Thai-CPIV-5 contained unique amino acids of Asian CPIV-5 subcluster and distinguished from W3A and CPIV-5 from the US and UK. Thus, it more likely that the CPIV-5 in this study were isolated from naturally infected dogs in Thailand.

To date, only nine whole genome sequences of CPIV-5 are available in the GenBank database. This study provided additional information on the whole genome sequences of CPIV-5 from Thailand (n=3). Based on phylogenetic analysis of the whole genome, Thai CPIV-5 belongs to parainfluenza type 5 and subcluster CPIV-5 (canine sublineage) and is separated from swine and human sublineage. Within the canine sublineage, Thai CPIV-5 was closely related to CPIV-5 from South Korea (08-

1990 and D227) and China (CPIV-HeN0718). Thai CPIV-5 had the highest nucleotide identities (99.4%) to CPIV-5 from Korea. Phylogenetic analyses of the HN, F, V/P, M, NP, and L genes showed similar results, in which Thai CPIV-5 was grouped together with CPIV-5 from Korea (08-1990 and D227) and China (CPIV-HeN0718). One Thai CPIV-5 (CU-D399) was closely related to PIV-5 from the pangolin (CAN) and lesser panda (ZJQ-221), which was similar to CPIV-5 (1168-1 from Korea). Our results suggested that Thai CPIV-5 potentially originated from the same ancestor as CPIV-5 from China and South Korea. Similarly, a unique cluster of CPIV-5 from dog in China (CC-1A, 2000s), PIV-5 from calf in China (PV5-BC14, 2014) and PIV-5 from pig in Germany (SER, 1998) and South Korea (KUN-11, 2011) was observed suggesting potential common ancestor of these viruses and required further investigations.

Thai CPIV-5 contained no amino acid mutations in the HN protein at the receptor binding site, cleavage site or HN stalk region. It has been reported that the amino acid residue at E37 is associated with virus entry into host cells by clathrin-coated pits and the endocytic pathway (Leser et al., 1996; Robach and Lamb, 2010). Amino acid residues at L90, E91, Q102, QDHVS (186-190), E390 and Y523 are associated with viral receptor binding of the viruses (Melanson and Iorio, 2004; Yuan et al., 2005). Amino acid residues at K342, T437, and F457 are associated with neutralizing antibodies (Baty and Randall, 1993). In this study, some Thai CPIV-5 contained T437I (CU-D58 and CU-D376) and K342Q (CU-D399) which is similar to PIV-5 from dogs and humans. However, the importance of these mutations (T437I and K342Q) in neutralizing antibodies requires further investigation. A previous study reported that mutations in HN stalk regions might affect viral fusion to host cells (Corey and Iorio, 2007; Yuan et al., 2005).

Thai CPIV-5 contained some host preference amino acid residues (human specific residues) in the F gene. For example, the amino acids at 22P and 443P in the F gene were observed in both Thai CPIV-5 and PIV-5 from humans and pigs (Bose et al., 2013; Ito et al., 2009; Rima et al., 2014). One Thai CPIV-5 (CU-D151) also contained R536Q, similar to human PIV-5. For the V and P proteins, there was no amino acid mutation in Thai PCIV-5. It has been reported that amino acid mutations of S157F, K254R and T286A of V and P protein can result in high progeny virus

production and the apoptosis of infected cells (Timani et al., 2008) (Sun et al., 2011a; Sun et al., 2011b; Timani et al., 2008). For the SH protein, Thai CPIV-5 contained an amino acid substitution at the start codon, which can also be observed in swine PIV-5, cattle PIV-5 and canine PIV-5. Mutation of the start codon can result in no expression of the SH protein (Chatziandreou et al., 2004; Rima et al., 2014). The function of the SH protein is unclear, but some studies have reported an association with virus survival in host cells and control of host cell apoptosis (He et al., 2001; Rima et al., 2014; Wilson et al., 2006). It should be noted that Thai, Chinese and Korean CPIV-5 contained four amino acids longer than the reference PIV-5. Thus, the SH gene can be used as a genetic marker for the differentiation of Asian CPIV-5 from other CPIV-5.

## 2.6 Conclusion

In summary, this study is the first report of whole genome characterization of CPIV-5 in Thailand. Phylogenetic analyses showed that Thai CPIV-5 might have originated from a common ancestor with CPIV-5 from Korea and China. To date, there is no evidence of PIV-5 cross-species transmission between dogs and humans. However, it is imperative to educate pet owners, veterinarians and others who come into close contact with domestic dogs about zoonotic awareness. In Thailand, the surveillance of CPIV-5 should be further investigated on a larger scale to determine the dynamics, distribution, and genetic characteristics of CPIV-5.

## CHAPTER III

### CANINE INFLUENZA VIRUS

The manuscript is in preparation in title

#### Characterization of pandemic H1N1-2009 in dogs, Thailand

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#### 3.1 Abstract

In this study, we conducted a survey of influenza A viruses in dogs in Thailand during November 2015 to December 2018. The nasal swab samples (n=571) were collected from dogs from small animal hospitals located in Bangkok, Thailand. The nasal swab samples were tested for influenza A virus by using real time RT-PCR specific to M gene of the virus. Our results showed that 1.23 % (7/571) samples were positive for influenza A virus. One influenza A viruses was successfully subtyped and characterized by whole genome sequencing. The virus was designated as pandemic H1N1-2009 influenza A virus. This study is the first report of the detection and characterization of pdmH1N1-2009 in dog in Thailand. Phylogenetic and genetic analysis of the canine pandemic H1N1-2009 characterized in this study showed that the Thai canine pdmH1N1-2009 was grouped with the pdmH1N1-2009 circulating in human. The genetic characteristics of the virus was resembled to those of the pdmH1N1-2009. Our results confirmed the evidence of reverse zoonosis of influenza virus from human to dog. Thus, human- dog interface is important for increasing the risk of cross-species transmission of influenza viruses. Risk communications and practices regarding to the risk of influenza virus infection among owners, veterinarians, or persons with close contact with animals should be provided especially in high human-dog interface settings.

**Keywords:** Characterization, dogs, pandemic H1N1-2009, Thailand

### 3.2. Introduction

Influenza virus infection has impacts on economy and public health worldwide. Influenza virus is a negative sense, single-stranded RNA virus and belongs to the Orthomyxoviridae family. Influenza virus can be divided into 4 types (A, B, C and D). Influenza A virus (IAV) can infect and causes respiratory diseases in several animal species and humans. IAV comprises with 8 genes encoding 11 proteins (HA, NA, NP, M1, M2, NS1, NS2, PA, PB1, PB1-F2 and PB2). Subtyping of IAVs is based on two surface envelop proteins, HA and NA. To date, 18 HA (H1-18) and 11 NA (N1-11) subtypes have been reported (Tong et al., 2013a).

Influenza A virus can also infect in dogs and causes canine influenza outbreaks in dogs in several countries. Infected dogs developed respiratory symptoms and possibly complications due to secondary bacterial infection including cough, nasal discharge, fever and pneumonia (Jung et al., 2010). In 2004, canine influenza subtype H3N8 (CIV-H3N8) of equine origin was first reported in racing greyhound dogs with respiratory disease in Florida, USA (Crawford et al., 2005b). In 2010-2012, there were reported canine influenza subtype H3N2 of avian-origin (CIV-H3N2) in dogs and this virus can also transmitted among dogs to dogs in several countries (Lin et al., 2012c, d; Song et al., 2011; Song et al., 2012b).

In 2009, pandemic outbreaks of pandemic H1N1/2009 (pdmH1N1-2009) have been reported worldwide (Patient, 2009). Consequently, there were several reported of spill over events of pdmH1N1/2009 in other mammals, including pigs, cats and dogs (Chastagner et al., 2018; Lin et al., 2012a; Pingret et al., 2010; Sponseller et al., 2010; Su et al., 2014). The persistent of pdmH1N1-2009 for decade in human and mammals are leading to the generation of novel or reassorted virus with high infectivity and virulence. The evidence of novel reassortant viruses between human and canine viruses were IAV subtype H3N1 and H3N2 in dogs (Moon et al., 2015; Na et al., 2015a; Song et al., 2012b). Since, the evidence of reassortment of pdmH1N1-2009 and canine influenza suggested that dogs play an important role as “mixing vessel” for generating novel subtypes or reassortment viruses (Chen et al., 2018). Thus, close contact between dogs and humans increase an opportunity of influenza infection and subsequently generating novel reassortant viruses (Parrish et al., 2015a;

Ramírez-Martínez et al., 2013).

In Thailand, IAV subtypes H5N1 and H3N2 had been reported to infect dogs with respiratory signs (Bunpapong et al., 2014a; Songserm et al., 2006). The prevalence of influenza in dogs with respiratory diseases had also been reported (Posuwan et al., 2010a). Interestingly, a serological survey showed that dogs in Thailand had antibodies against to both canine influenza (H3N2) and human influenza (H3N2, pdmH1N1) (Chanvatick et al., 2016; Tangwangvivat et al., 2019). Up to date, they were limited genetic information of influenza A viruses in dogs. In Thailand, this study aimed to conducted IAV survey in dogs and genetic characterized IAVs in dogs by whole genome sequencing.





### 3.3. Materials and Methods Canine Samples

#### 3.3.1 Dog samples

In this study, cross-sectional samples collection was conducted at the Chulalongkorn University's Veterinary Teaching hospital and private small animal hospitals during November 2015 to December 2018. A total of 578 nasal swab samples were collected from dogs with respiratory symptoms including sneezing, nasal discharge, cough, and dyspnea. The animal demographic data including age, sex, breed, contact history, and vaccination history were recorded. The Chulalongkorn University, Animal Care and Uses Protocol committee approved the animal study (CU-VET IACUC #1731074). All animal study procedures were performed in accordance with CU-VET IACUC guidelines and regulations.

#### 3.3.2 Influenza A Virus identification

All nasal swab samples were subjected to RNA extraction by using the QIAmp viral RNA mini kit (Qiagen, Hilden, Germany) following manufacturer's recommendations. Briefly, the 140  $\mu$ l of nasal swab sample was lysed by Buffer AVL-carrier RNA and 560  $\mu$ l of ethanol. Following, the mixture was centrifuged and moved into column then the 500  $\mu$ l of buffer AW1 and AW2 were added to wash away the contaminants from the sample, Finally the RNA was eluted by 50  $\mu$ l of buffer AVE. RNA was stored at  $-20^{\circ}\text{C}$  until use. To detect influenza A virus, RNA sample was screened for influenza A virus by using one-step Real-time RT-PCR assay with a TaqMan probe specific for the matrix (M) gene. The primers and probe used in this study were previously described including IAV- M+25: 5'- AGATGAGTCTTCTAA CCGAGGTCG, IAV-124: 5'- TGCAAAAACATCTTCAAGTCT, probe M+64: 5'FAM- TCA GGCCCCCTCAAAGCCGA-TAMRA (Spackman et al., 2002). Briefly, one-step Real time RT-PCR (Invitrogen, USA) was conducted in a total final volume of 25  $\mu$ l comprising 4  $\mu$ l of template RNA, 12.5  $\mu$ l of 2X reaction buffer of the SuperScript® III Platinum® One-Step Quantitative, 0.4  $\mu$ M of each primer, 1  $\mu$ l of reverse transcriptase/Platinum Taq, 0.4 mM of  $\text{MgSO}_4$ , 0.4  $\mu$ M of probe and RNase-free water.

For one -step real time RT-PCR condition, the reaction contained cDNA

synthesis step at 55°C for 10 min, followed by 95°C for 3 min and then 45 cycles of 95°C for 15 s, 58°C for 30 s. Real-time RT-PCR result was interpreted by cycle threshold (Ct- value) of <36 as positive, Ct-value of 36–40 as suspected, and Ct-value of >40 as negative.

For influenza A virus isolation, the real time positive samples (n=7) were subjected to viral isolation by embryonated chicken egg inoculation and MDCK (Madin-Darby canine kidney) cell culture following WHO recommendation. For embryonated chicken egg inoculation, 100 µl of supernatant of nasal swab sample was inoculated into 3 embryonated chicken eggs and incubated at 37 °C for 72 hours. Allantois fluid was harvested and screened for hemagglutinating activity by HA test. Sample with HA titer of  $\geq 4$  HA unit per 50 µl was interpret as positive. For virus confirmation, RNA sample was tested for influenzas A virus by using one-step Real-time RT-PCR. For MDCK (Madin-Darby canine kidney) cell culture, the supernatant of nasal swab sample was inoculated into MDCK in Minimal Essential Medium (MEM, Gibco, by Life Technologies) with 10% fetal bovine serum (Gibco, by Life Technologies), 100 U/ml Penicillin and 0.5 mg/ml Streptomycin (Hi-Media Laboratories, India). Tissue culture fluid was harvested after observing cytopathic effect (WHO, 2002).

### 3.3.3. Influenza A virus characterization

In this study, all IAVs were selected for whole genome sequencing by next-generation sequencing (NGS). The all 8 gene segments of IAVs were amplified by using one-step RT-PCR with SuperScript III RT-PCR system with Platinum Taq DNA polymerase (Invitrogen; California, USA) by using MBT12 and MBT13 primers (Zhou et al., 2009). Purified PCR products were submitted to Novogene co. LTD for Illumina Hiseq PE150 (Illumina Corporation, San Diego, California, USA) by using NEBNext Multiplex Oligos for Illumina (New England BioLabs, Ipswich, Massachusetts, USA). The sequence validation determination and assembly of eight genes were done by using de-novo assembly method by CLC genomics workbench software Version 11.0.1. (QIAGEN; Hilden, Germany). The sequence contigs were compared with sequence database by BLAST. After the references influenzas viruses were selected

from BLAST results, the trimmed sequences were used for reading mapping to references. Finally, the whole genome sequences were extracted to FASTA format (.fas) by CLC genomics workbench software.

The phylogenetic analysis of IAV was performed by using MEGA v.6.0 (Tempe, AZ, USA) (Tamura et al., 2013) with neighbor-joining method with Kimura 2-parameter with 1000 bootstrap replications. For genetic analysis, the nucleotide sequences and deduced amino acids of IAVs were aligned and compared using MegAlign software v.5.03 (DNASTAR Inc.; Wisconsin, USA). In this study, nucleotide sequences of the CIV were submitted to the GenBank database under the accession numbers (Table 3.1).



**Table 3.1.** Description of influenza A virus detected and characterized in this study.

Virus	Date of collection	Age	Breed	Ct-value	Sequencing	GenBank accession number
AD 103	Feb 16	2 Months	Bully	32	N/A	N/A
AD 104	Feb 16	2 Months	Mixed	31.5	N/A	N/A
AD 105	Feb-16	7 year	Chihuahua	31.3	WGS	N/A
AD 580	Aug-17	5 Months	Mixed	36.28	N/A	N/A
AD 582	Sep-17	9 years	Pug	32.997	N/A	N/A
AD 586	Sep-17	3 Months	Mixed	31.723	N/A	N/A
AD 587	Sep-17	3 Months	French bulldog	32.7	N/A	N/A



### 3.4 Results

In this study, we investigated influenza A virus (IAV) infection in dogs with respiratory symptoms during November 2015 to December 2018. Our result showed that 1.23 % (7/571) of nasal swab samples were positive for IAV (Table 3.1). From 3-year surveillance, highest occurrence of IAV is observed in winter seasons (Table 3.2). In relation between IAV infection to age group, occurrence of IAV by age group were 1.26% (5/396) in dogs <1 year, and 2.53% (2/79) in dogs older than 6 years (data not shown).

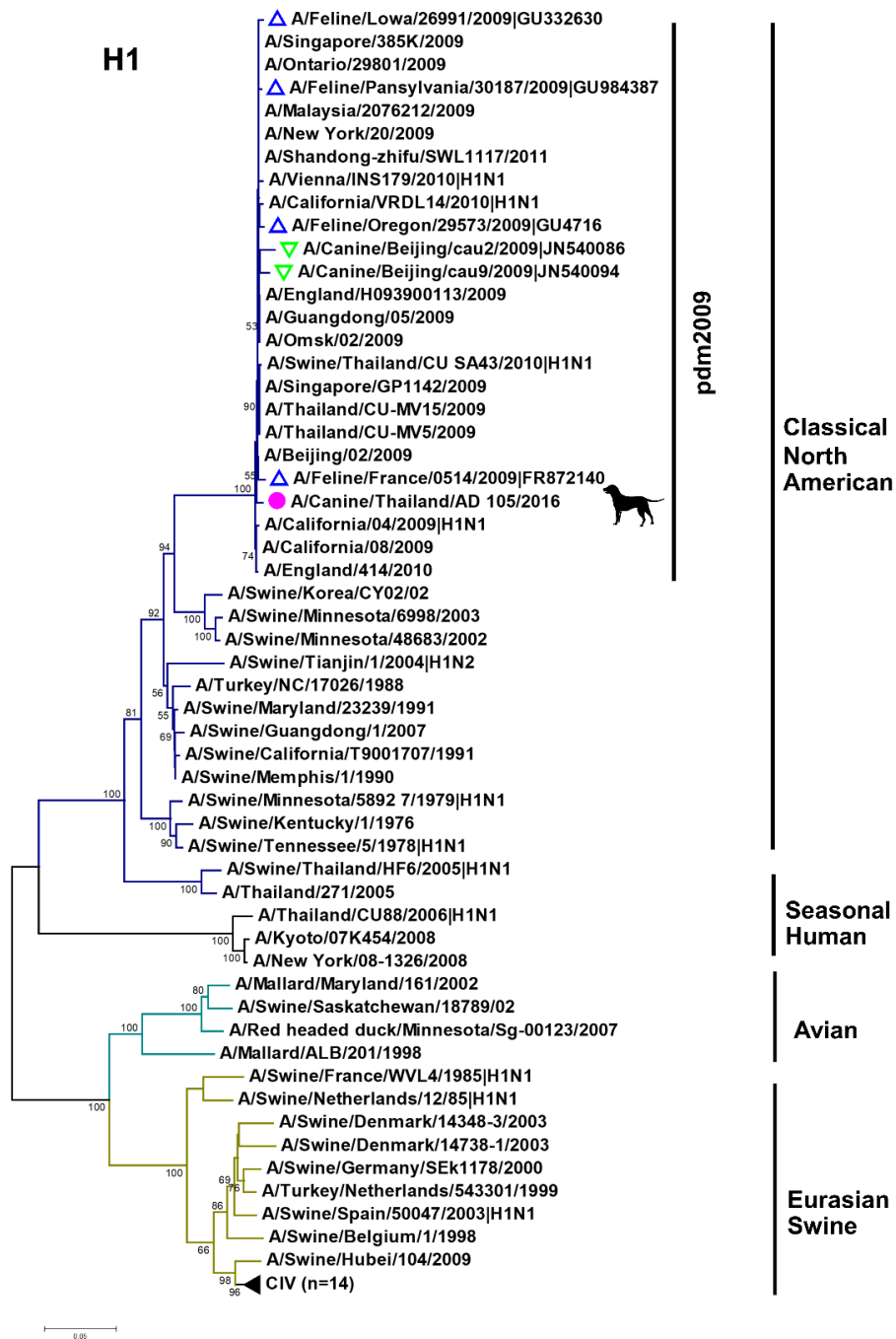


**Table 3.2.** Detail of samples collection and detection of influenza A virus

Year	Month	Total sample	Number of positive IVA (%)
2015	Nov	7	0
	Dec	10	0
2016	Jan	9	0
	Feb	11	3 (27.27%)
	Mar	7	0
	Apr	13	0
	May	12	0
	June	3	0
	July	15	0
	Aug	10	0
	Sep	9	0
	Oct	11	0
	Nov	12	0
	Dec	15	0
2017	Jan	12	0
	Feb	26	0
	Mar	32	0
	Apr	24	0
	May	11	0
	June	11	0
	July	19	0
	Aug	17	1 (5.88%)
	Sep	23	3 (13.04%)
	Oct	21	0
	Nov	13	0
	Dec	33	0
2018	Jan	19	0
	Feb	17	0
	Mar	14	0
	Apr	18	0
	May	9	0
	June	19	0
	July	21	0
	Aug	18	0
	Sep	11	0
	Oct	10	0
	Nov	15	0
	Dec	14	0
<b>Total</b>		<b>571</b>	<b>7 (1.23%)</b>

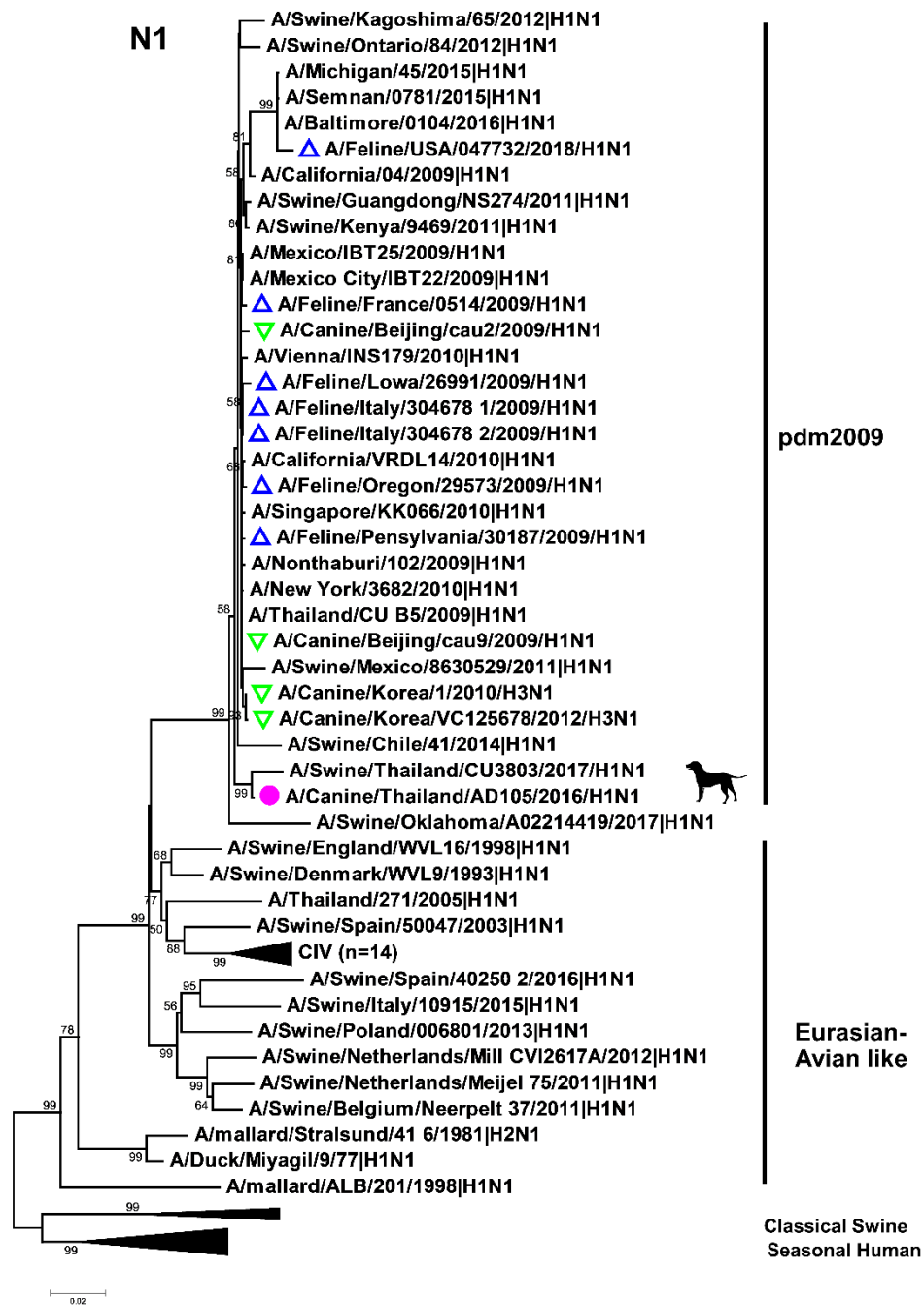
### 3.4.1. Genetic characteristics of influenza A virus from dogs

In this study, real-time RT-PCR positive samples (n=7) were subjected to virus isolation by egg inoculation and/or cell culture. All the supernatants were negative for HA test, CPE formation. Moreover, the real-time RT-PCR result of these supernatants (n=7) were also negative. However, the RNA from original nasal swab samples were tested positive and subjected to whole genome sequencing by Illumina Miseq Next generation sequencing. 1 out of 7 samples (AD 105; Ct-value 31.3) was successfully sequence and whole genome sequence of the virus was achieved. Based on the whole genome sequence analysis, our results showed that IAV from dog (AD105) was identified as pdmH1N1-2009. The phylogenetic tree of H1 gene was constructed by comparing the reference IAVs from dogs, cats, pigs, avian and humans. The phylogenetic tree of H1, Thai pdmH1N1-2009 (AD105) was grouped with the pdmH1-2009 of the classical lineage which same cluster with pdmH1-2009 in dogs from China (Cau2, Cau9) and cats (0514, 29573) from France and USA (Figure 3.1). The phylogenetic tree of N1 gene, Thai pdmH1N1-2009 (AD105) was clustered with pdmH1N1-2009 in pigs from Thailand (CU3803), dog from China and South Korea (Korea/1, Cau2 and Cau9) and cat from France, Italy, and USA (Figure 3.2). Phylogenetic trees of the internal genes of Thai-pdmH1N1-2009 (AD105) were constructed by comparing the viruses with reference influenza viruses from dogs, cats, pigs and human. Phylogenetic analysis of each internal genes showed that internal genes of Thai pdmH1N1-2009 (AD105) were clustered with the viruses of pdm09 lineage confirming that Thai pdmH1N1-2009 in dog in this study contained all 8 genes of pdm/09 (Figures 3.3–3.8). Genetic constellation of Thai pdmH1N1-2009 in this study was performed by using combination of eight lineages of virus. The 8 genes of Thai pdmH1N1-2009 (AD105) composed genes originated from pdm/09 which is similar to those of viruses from dog in China (Cau2, Cau9), cats from France and USA (0514, 29573) (Table 3.3 and Table 3.4). Moreover, all gene segments of Thai pdmH1N1-2009 (AD105) possessed high nucleotide identities to pdm/H1N1/2009 from dogs in China (Cau2, Cau9) and cat in France (France/0514) with 96.00-99.70% nucleotide identities (96.40-100.00 % amino acid identities) (Table 3.4).



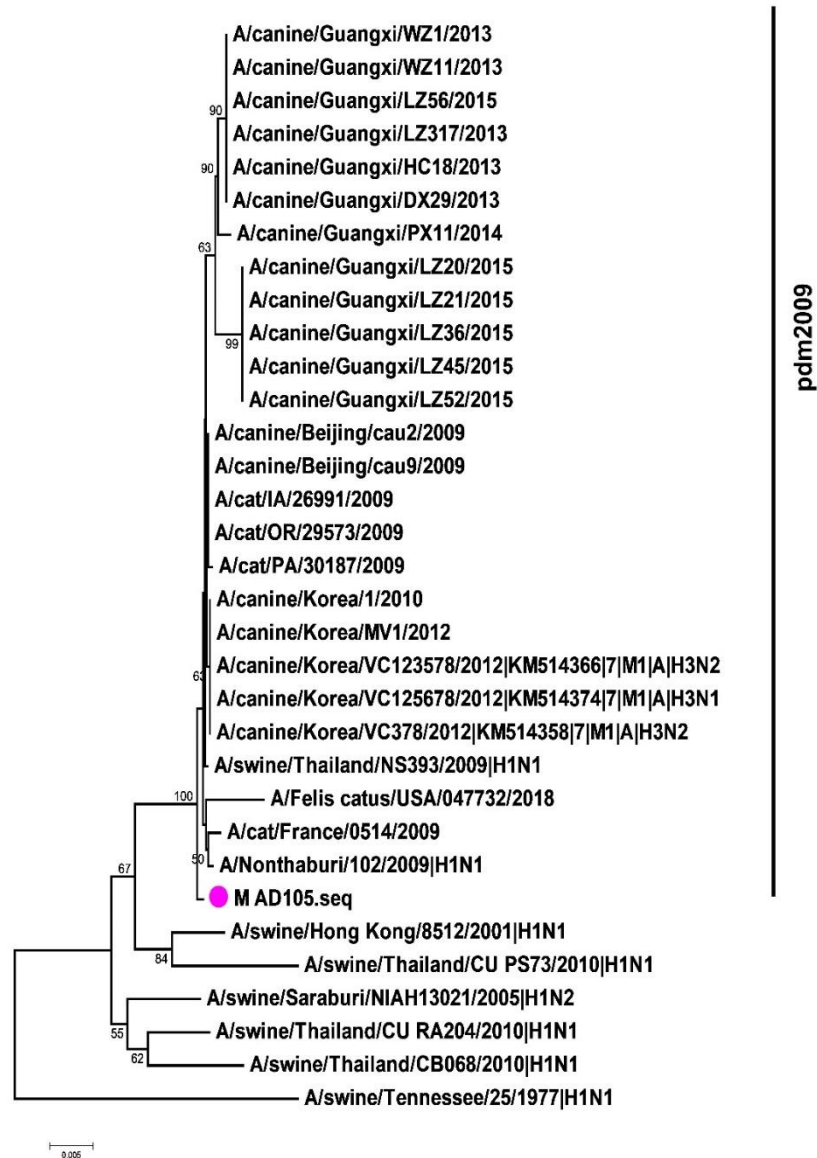
**Figure 3.1.** Phylogenetic tree of H1 gene. The phylogenetic tree was constructed by using the neighbor-joining algorithm with kimura-2 parameter model with 1000 replication bootstrap. The pink symbol indicates Thai pdmH1N1-2009 in this study. The blue triangular are feline pdmH1N1-2009 from cats. The green triangular are pdmH1N1-2009 from dogs.





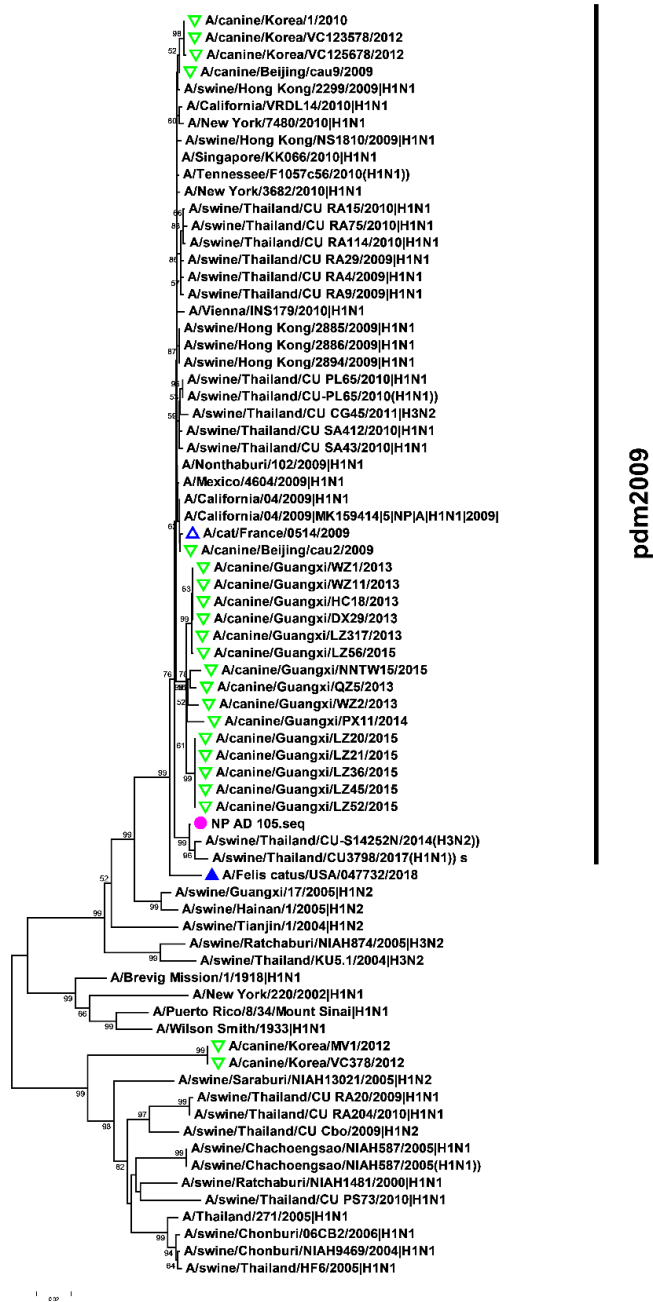
**Figure 3.2.** Phylogenetic tree of N1 gene. The phylogenetic tree was constructed by using the neighbor-joining algorithm with kimura-2 parameter model with 1000 replication bootstrap. The pink symbol indicates Thai pdmH1N1-2009 in this study. The blue triangular are feline pdmH1N1-2009 from cats. The green triangular are pdmH1N1-2009 from dogs.

M

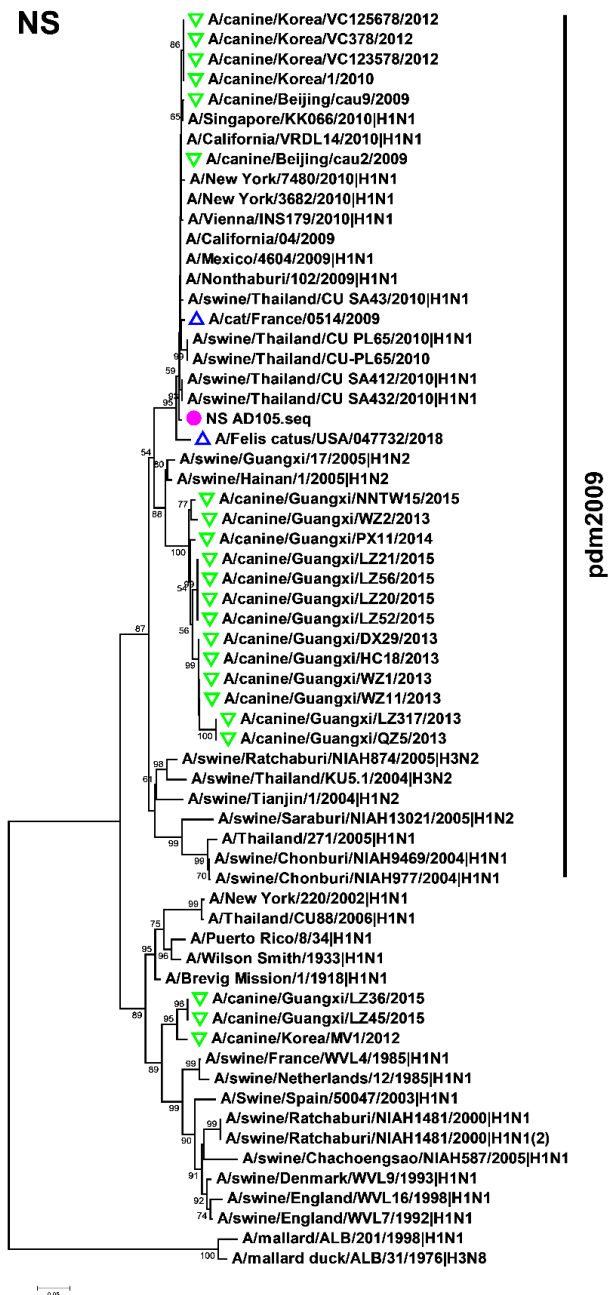


**Figure 3.3.** Phylogenetic tree of M gene. The phylogenetic tree was constructed by using the neighbor-joining algorithm with kimura-2 parameter model with 1000 replication bootstrap. The pink symbol indicates Thai pdmH1N1-2009 in this study.

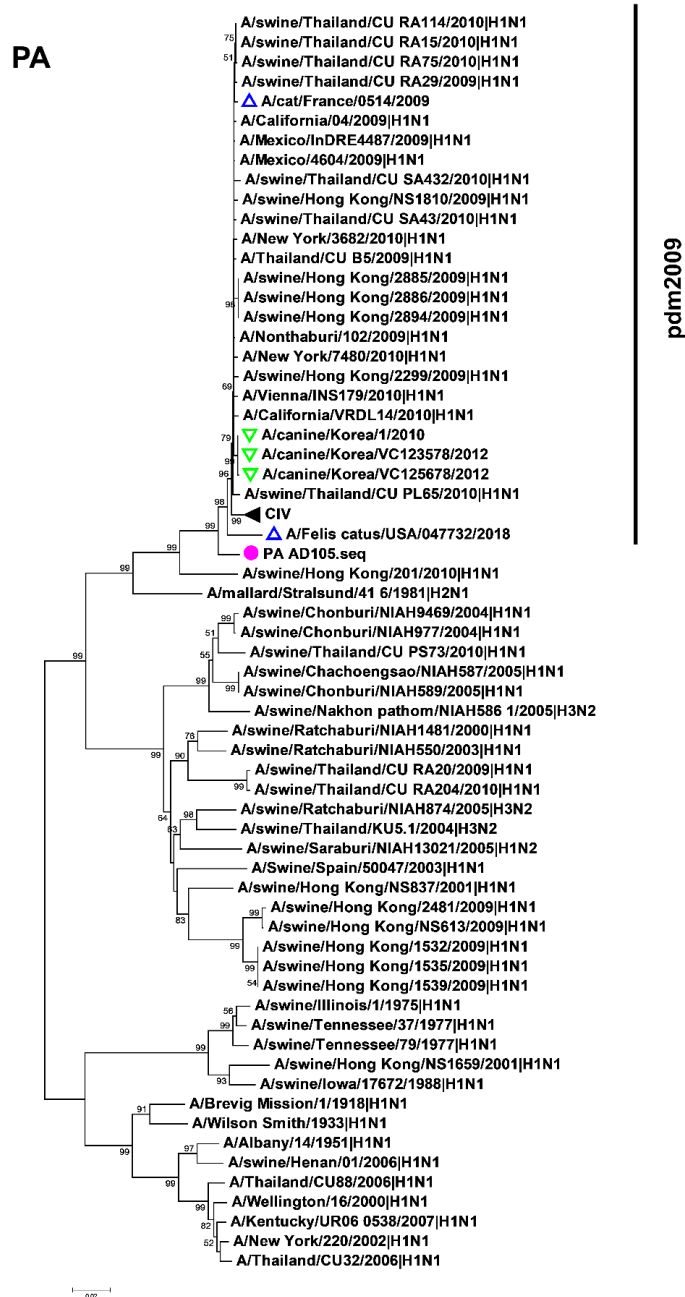
NP



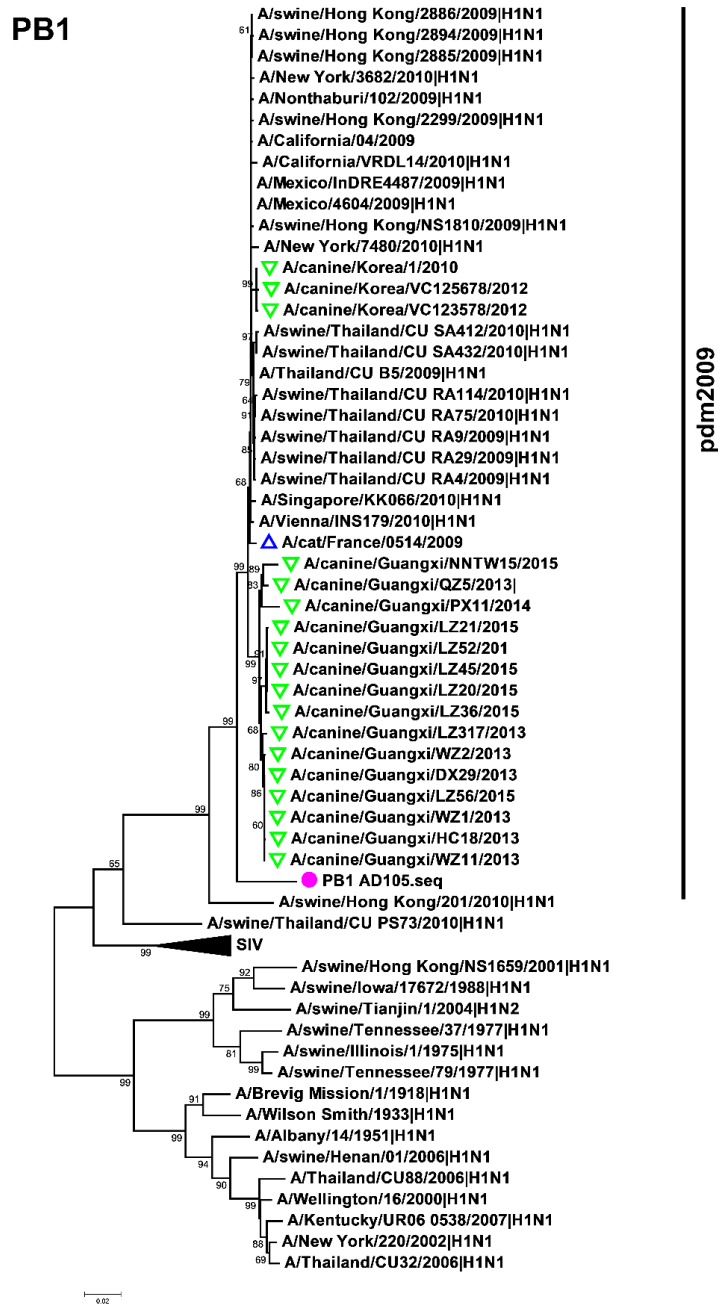
**Figure 3.4.** Phylogenetic tree of NP gene. The phylogenetic tree was constructed by using the neighbor-joining algorithm with kimura-2 parameter model with 1000 replication bootstrap. The pink symbol indicates Thai pdmH1N1-2009 in this study. The blue triangular are feline pdmH1N1-2009 from cats. The green triangular are pdmH1N1-2009 from dogs.



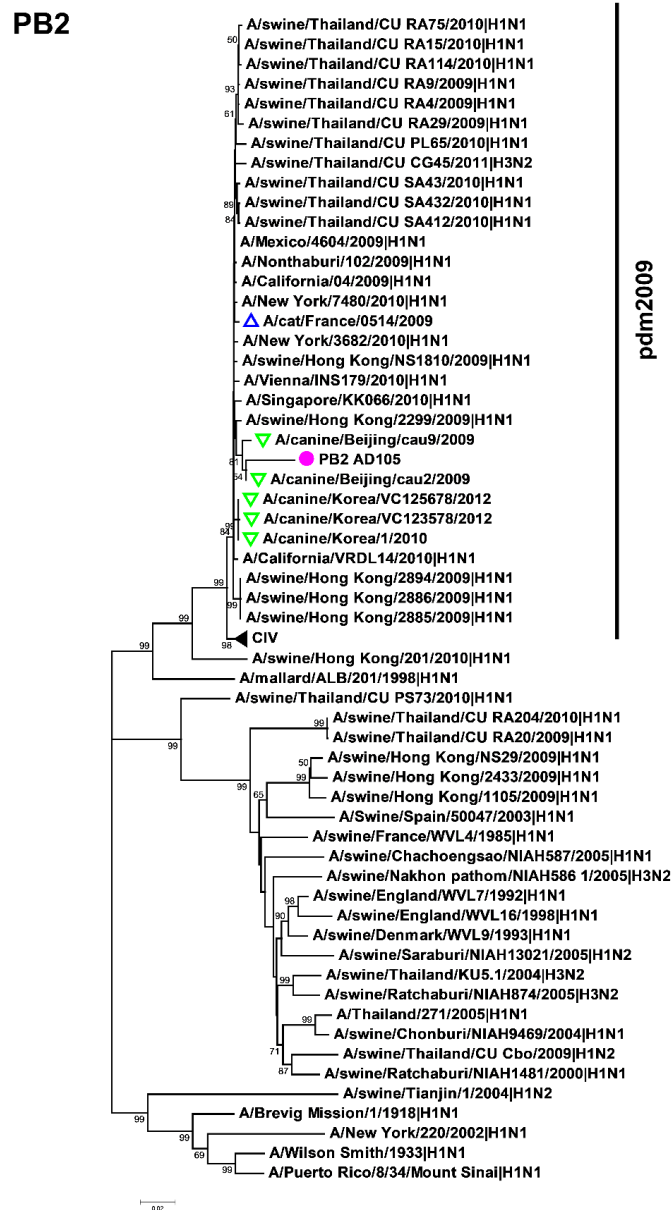
**Figure 3.5.** Phylogenetic tree of NS gene. The phylogenetic tree was constructed by using the neighbor-joining algorithm with kimura-2 parameter model with 1000 replication bootstrap. The pink symbol indicates Thai pdmH1N1-2009 in this study. The blue triangular are feline pdmH1N1-2009 from cats. The green triangular are pdmH1N1-2009 from dogs.



**Figure 3.6.** Phylogenetic tree of PA gene. The phylogenetic tree was constructed by using the neighbor-joining algorithm with kimura-2 parameter model with 1000 replication bootstrap. The pink symbol indicates Thai pdmH1N1-2009 in this study. The blue triangular are feline pdmH1N1-2009 from cats. The green triangular are pdmH1N1-2009 from dogs.



**Figure 3.7.** Phylogenetic tree of PB1 gene. The phylogenetic tree was constructed by using the neighbor-joining algorithm with kimura-2 parameter model with 1000 replication bootstrap. The pink symbol indicates Thai pdmH1N1-2009 in this study. The blue triangular are feline pdmH1N1-2009 from cats. The green triangular are pdmH1N1-2009 from dogs.



**Figure 3.8.** Phylogenetic tree of PB2 gene. The phylogenetic tree was constructed by using the neighbor-joining algorithm with kimura-2 parameter model with 1000 replication bootstrap. The pink symbol indicates Thai pdmH1N1-2009 in this study. The blue triangular are feline pdmH1N1-2009 from cats. The green triangular are pdmH1N1-2009 from dogs.





**Table 3.4.** Pairwise comparisons of nucleotide and amino acid of Thai pdmH1N1-2009 (AD105) from dog with those of reference influenza viruses.

Viruses	Year	Country	Nucleotide (amino acid) identity, %										
			PB2	PB1	PA	HA	NP	NA	M	NS			
<b>This study</b>													
AD105	2016	Thailand	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	
<b>Reference</b>													
California/04	2009	USA	98.00 (97.80)	96.10 (96.60)	98.60 (99.60)	99.20 (99.10)	98.80 (99.60)	98.80 (99.80)	99.70 (99.60)	99.70 (99.60)	99.40 (99.10)	99.40 (99.10)	
THA/CUPL65	2010	Thailand	95.80 (95.90)	93.80 (94.20)	97.8 (99.00)	98.30 (98.20)	98.70 (100.00)	94.00 (94.70)	98.90 (98.40)	98.90 (98.40)	98.30 (98.20)	98.30 (98.20)	
THA/NAH587	2005	Thailand	82.80 (93.30)	84.60 (92.90)	86.20 (94.40)	85.00 (87.50)	82.90 (92.50)	90.10 (92.10)	95.40 (98.00)	95.40 (98.00)	79.10 (77.20)	79.10 (77.20)	
<b>Canine H1 reference</b>													
Beijing/cau2	2009	China	98.40 <sup>a</sup> (98.30)	96.60 <sup>b</sup> (99.10)	98.30 <sup>c</sup> (98.10)	98.40 (97.90)	98.80 <sup>f</sup> (100.00)	98.50 (99.30)	99.70 (99.60)	99.70 (99.60)	99.20 (98.60)	99.20 (98.60)	
Beijing/cau9	2009	China	98.30 <sup>a</sup> (98.30)	96.70 <sup>b</sup> (99.50)	98.30 <sup>c</sup> (98.70)	98.60 (97.50)	99.00 <sup>f</sup> (100.00)	98.7 (99.30)	99.70 (99.60)	99.70 (99.60)	98.90 (97.70)	98.90 (97.70)	
Guangxi/DX29	2013	China	97.10 (97.20)	95.30 (96.20)	97.30 (98.70)	74.20 (78.60)	98.10 (99.60)	89.80 (91.80)	99.10 (99.20)	99.10 (99.20)	90.30 (87.70)	90.30 (87.70)	
Guangxi/Hc18	2013	China	97.20 (97.20)	95.20 (96.20)	97.30 (98.60)	74.20 (78.60)	98.10 (99.60)	89.90 (91.80)	99.10 (99.20)	99.10 (99.20)	90.30 (87.70)	90.30 (87.70)	
Guangxi/LZ20	2015	China	96.90 (97.20)	95.00 (95.80)	96.70 (98.70)	74.00 (78.80)	98.10 (99.60)	89.60 (91.80)	98.70 (99.60)	98.70 (99.60)	90.70 (88.60)	90.70 (88.60)	
Guangxi/LZ21	2015	China	96.90 (97.20)	95.00 (95.60)	96.70 (98.70)	74.00 (78.80)	98.10 (99.60)	89.60 (91.80)	98.70 (99.60)	98.70 (99.60)	90.70 (88.60)	90.70 (88.60)	
Guangxi/LZ317	2013	China	97.20 (97.10)	95.00 (95.50)	97.20 (98.70)	74.00 (78.40)	98.10 (99.80)	89.80 (91.80)	99.10 (99.20)	99.10 (99.20)	88.30 (84.50)	88.30 (84.50)	
Guangxi/LZ36	2015	China	96.90 (97.20)	94.80 (95.90)	96.60 (98.70)	74.00 (78.80)	98.10 (99.60)	89.60 (91.80)	98.70 (99.60)	98.70 (99.60)	84.00 (83.60)	84.00 (83.60)	
Guangxi/LZ52	2015	China	96.90 (97.20)	95.00 (95.80)	96.70 (98.70)	73.90 (78.60)	98.10 (99.60)	89.60 (91.80)	98.70 (99.60)	98.70 (99.60)	90.60 (88.10)	90.60 (88.10)	
Guangxi/LZ56	2015	China	97.30 (97.40)	95.30 (96.20)	97.30 (98.70)	74.00 (78.40)	98.00 (99.60)	89.90 (91.80)	99.10 (99.20)	99.10 (99.20)	90.70 (88.60)	90.70 (88.60)	
Guangxi/NNTW15	2015	China	96.80 (96.70)	94.80 (96.20)	97.10 (98.50)	74.00 (78.80)	97.70 (99.00)	89.50 (91.20)	94.70 (98.00)	94.70 (98.00)	90.70 (89.00)	90.70 (89.00)	
Guangxi/QZ5	2013	China	97.20 (97.20)	95.00 (96.00)	97.50 (99.20)	74.30 (78.40)	97.90 (99.20)	89.40 (91.40)	94.70 (98.00)	94.70 (98.00)	88.30 (84.50)	88.30 (84.50)	
Guangxi/WZ1	2013	China	97.10 (97.20)	95.30 (96.20)	97.30 (98.70)	74.10 (78.60)	98.10 (99.60)	89.60 (91.60)	99.10 (99.20)	99.10 (99.20)	90.30 (87.70)	90.30 (87.70)	
Guangxi/WZ11	2013	China	97.10 (97.20)	95.30 (96.20)	97.30 (98.70)	74.20 (78.60)	98.10 (99.60)	89.60 (91.40)	99.10 (99.20)	99.10 (99.20)	90.30 (87.70)	90.30 (87.70)	
Guangxi/WZ2	2013	China	N/A	95.20 (96.20)	97.20 (98.60)	74.30 (78.40)	97.70 (99.60)	89.30 (91.40)	95.10 (98.40)	95.10 (98.40)	90.40 (88.10)	90.40 (88.10)	
<b>Canine H3 reference</b>													
Korea/1/2010/H3N1	2010	Korea	98.00 (97.80)	95.90 (96.40)	98.40 (99.40)	51.30 (44.30)	98.50 (99.40)	98.50 (98.90)	99.60 (99.60)	99.60 (99.60)	98.90 (97.70)	98.90 (97.70)	
CU-DC5299/H3N2		Thailand	83.50 (95.7)	85.90 (92.90)	90.90 (96.10)	51.40 (44.30)	82.10 (92.90)	52.30 (29.40)	90.30 (94.90)	90.30 (94.90)	83.90 (83.10)	83.90 (83.10)	
<b>Feline H1 reference</b>													
France/0514	2009	France	97.90 (97.80)	96.00 (96.40)	98.50 (99.60)	98.90 (99.30)	98.60 (99.60)	98.50 (99.60)	99.30 (99.60)	99.30 (99.60)	98.80 (97.30)	98.80 (97.30)	
Iowa/26991	2009	USA	N/A	N/A	N/A	99.10 (99.10)	N/A	98.20 (99.10)	99.70 (99.60)	99.70 (99.60)	N/A	N/A	
Oregon/29573	2009	USA	N/A	N/A	N/A	99.20 (99.30)	N/A	98.60 (99.10)	99.70 (99.60)	99.70 (99.60)	N/A	N/A	
Pansylvanlar/30187	2009	USA	N/A	N/A	N/A	99.20 (99.30)	N/A	98.50 (99.30)	99.60 (99.60)	99.60 (99.60)	N/A	N/A	
046632	2018	USA	95.10 (95.70)	N/A	95.80 (97.80)	96.00 (95.50)	96.50 (99.20)	95.90 (95.40)	97.80 (97.20)	97.80 (97.20)	96.80 (96.30)	96.80 (96.30)	
304678_1	2009	Italy	N/A	N/A	N/A	99.10 <sup>a</sup> (98.80)	N/A	98.60 (99.30)	N/A	N/A	N/A	N/A	
304678_2	2009	Italy	N/A	N/A	N/A	99.10 <sup>a</sup> (98.80)	N/A	98.60 (99.30)	N/A	N/A	N/A	N/A	

### 3.4.2. Genetic analysis of Thai pdmH1N1-2009 (AD105)

Genetic analyses of the nucleotides and deduced amino acids of the Thai pdmH1N1-2009 (AD105) were conducted by comparing with the reference IVAs from dogs, cats, pigs, and humans. At the H1 cleavage site (325-333) of pdmH1N1-2009 (AD105) was contained “PSIQSRGLF” which similar to other reference pdmH1N1-2009 from dogs, cats and human. For receptor binding sites (HA-190, 225), Thai pdmH1N1-2009 (AD105) contained 190D and 225D which similar to IVAs from human and cat suggesting there are preferring to binding to human receptor (2,6 sialic acid receptor) (Connor et al., 1994). Genetic analysis of H1 antigenic sites (Sa, Sb, Ca1, Ca2 and Cb) showed that Thai pdmH1N1-2009 (AD105) contained identical amino acids at the antigenic sites to human, pig and cat (Table 3.5). For the PB2 gene, Thai pdmH1N1-2009 (AD105) contained amino acid substitutions at E267, 701D which are also observed in reference IVAs from dog, cat, pig and human suggesting correlated to the more virulence of viruses (Gao et al., 2009; Hatta et al., 2001; Steel et al., 2009). The NS gene, Thai pdmH1N1-2009 (AD105) possessed 92D. For antiviral resistance (oseltamivir) in NA1 gene, Thai pdmH1N1-2009 (AD105) contained E119, H275, R293, N295 which identical to other reference pdm H1N1/09 from cat, dog and human suggesting the viruses were sensitive to oseltamivir (Table 3.6) (Baek et al., 2015; Takashita et al., 2015).

Table 3.5. Genetic analysis of the H1 gene of Thai pdmH1N1-2009 (AD105) from dog.

Virus	H1 cluster	Year	Accession #	H1 gene*		Antigenic site					Receptor binding site		HA cleavage site							
				Sa	Sb	Ca1	Ca2	Cb	Ca1	Ca2	Cb	Ca1		Ca2	Cb					
<b>This study</b>																				
AD105	Pdm	2016		PN	KKGN	156-160	PKLSK	162-167	TSADQOQLYONA	187-198	INDKG	GSS	EPG	PHAGAK	RD	SILSTAS	D	D	PSIOSRGLF	
<b>Reference</b>																				
California/04	Pdm	2009	GOJ17044	PN	KKGN		PKLSK		TSADQOQLYONA		INDKG	GSS	EPG	PHAGAK	RD	SILSTAS	D	D	PSIOSRGLF	
THA/CUPL65	Pdm	2010	CY089797	PN	KKGN		PKLSK		TSADQOQLYONA		INDKG	GSS	EPG	PHAGAK	RD	SILSTAS	D	D	PSIOSRGLF	
THA/VIAH587	CS	2005	AB434328	PN	KKGN		PKLSK		TNTDQOQLYONA		VNKKK	GSS	EPG	PHAGAK	RG	LLFANN	D	G	PSIOSRGLF	
<b>Canine H1 reference</b>																				
Beijing/can2	Pdm	2009	JN540086	PN	KKGN		PKLSK		TDSDQOQLYONN		TNNKG	GSS	DOG	SHSGAN	RE	SILLTAN	D	E	PSIOSRGLF	
Beijing/can9	Pdm	2009	JN540094	PN	KKGN		PKLSK		TDSDQOQLYONK		TNNKG	GSS	DOG	SHSGAN	RE	SILLTAN	D	E	PSIOSRGLF	
Guangxi/DX29	CS	2013	MG254059	PN	KKGN		PKLSK		TDSDQOQLYONN		TNNKG	GSS	DOG	SHSGAN	RE	SILLTAN	D	E	PSIOSRGLF	
Guangxi/HC18	CS	2013	MG254060	PN	KKGN		PKLSK		TDSDQOQLYONN		TNNKG	GSS	DOG	SHSGAN	RE	SILLTAN	D	E	PSIOSRGLF	
Guangxi/LZ20	CS	2015	MG254066	PN	KKGN		PKLSK		TDSDQOQLYONN		TNNKG	GSS	DOG	SHSGAN	RE	SILLTAN	D	E	PSIOSRGLF	
Guangxi/LZ21	CS	2015	MG254067	PN	KKGN		PKLSK		TDSDQOQLYONN		TNNKG	GSS	DOG	SHSGAN	RE	SILLTAN	D	E	PSIOSRGLF	
Guangxi/LZ317	CS	2013	MG254072	PN	KKGN		PKLSK		TDSDQOQLYONN		TNNKG	GSS	DOG	SHSGAN	RE	SILLTAN	D	E	PSIOSRGLF	
Guangxi/LZ36	CS	2015	MG254068	PN	KKGN		PKLSK		TDSDQOQLYONN		TNNKG	GSS	DOG	SHSGAN	RG	SILLTAN	D	G	PSIOSRGLF	
Guangxi/LZ52	CS	2015	MG254070	PN	KKGN		PKLSK		TDSDQOQLYONN		TNNKG	GSS	DOG	SHSGAN	RE	SILLTAN	D	E	PSIOSRGLF	
Guangxi/LZ56	CS	2015	MG254071	PN	KKGN		PKLSK		TDSDQOQLYONN		TNNKG	GSS	DOG	SHSGAN	RE	LLLTAN	D	E	PSIOSRGLF	
Guangxi/NN45	CS	2014	MG254065	PN	KKGN		PKLSK		TDSDQOQLYONN		TNNKG	GSS	DOG	SHSGAN	RE	LLLTAN	D	E	PSIOSRGLF	
Guangxi/NN1W15	CS	2015	MG254073	PN	KKGN		PKLSK		TDSDQOQLYONN		TNNKG	GSS	DOG	SHSGAN	RE	SILLTAN	D	E	PSIOSRGLF	
Guangxi/OZ5	CS	2013	MG254061	PN	KKGN		PKLSK		TDSDQOQLYONN		TNNKG	GSS	DOG	SHSGAN	RE	SILLTAN	D	E	PSIOSRGLF	
Guangxi/WZ1	CS	2013	MG254062	PN	KKGN		PKLSK		TDSDQOQLYONN		TNNKG	GSS	DOG	SHSGAN	RE	LLLTAN	D	E	PSIOSRGLF	
Guangxi/WZ11	CS	2013	MG254064	PN	KKGN		PKLSK		TSADQOQLYONA		INDKG	GTS	EPG	PHAGAK	RD	SILSTAS	D	D	PSIOSRGLF	
Guangxi/WZ2	CS	2013	MG254063	PN	KKGN		PKLSK		TSADQOQLYONA		INDKG	GTS	EPG	PHAGAK	RD	SILSTAS	D	D	PSIOSRGLF	
<b>Feline H1 reference</b>																				
France/0514	Pdm	2009	FR872140	PN	KKGN		PKLSK		TSADQOQLYONA		INDKG	GTS	EPG	PHAGEK	RD	SILSTAS	D	D	PSIOSRGLF	
Lowia/26991	Pdm	2009	GU332630	PN	KKGN		PKLSK		TSADQOQLYONA		INDKG	GTS	EPG	PHAGAK	RD	SILSTAS	D	D	PSIOSRGLF	
Oregon/29573	Pdm	2009	GU4716	PN	KKGN		PKLSK		TSADQOQLYONA		INDKG	GTS	EPG	PHAGAK	RD	SILSTAS	D	D	PSIOSRGLF	
Pansyvania/30187	Pdm	2009	GU984387	PN	KKGN		PKLSK		TSADQOQLYONA		INDKG	GSS	EPG	PHAGAK	RD	SILSTAS	D	D	PSIOSRGLF	

\*Amino acid positions are based on H3 numbering.

**Table 3.6.** Genetic analysis of the internal genes of Thai pdmH1N1-2009 (AD105) from dog.

Viruses	Year	Country	Pb2		NS		NA		
			627	701	92	119	275	293	295
<b>This study</b>									
AD105	2016		E	D	D	E	H	R	N
<b>Reference</b>									
California/04	2009	USA	E	D	D	E	H	R	N
THA/CUPL65	2010	Thailand	E	D	D	E	H	R	N
THA/NIAH587	2005	Thailand	E	N	E	E	H	R	N
<b>Canine H1 reference</b>									
Beijing/cau2	2009	China	E	D	D	E	H	R	N
Beijing/cau9	2009	China	E	D	D	E	H	R	N
Guangxi/DX29	2013	China	E	D	D	E	H	R	N
Guangxi/HC18	2013	China	E	D	D	E	H	R	N
Guangxi/LZ20	2015	China	E	D	D	E	H	R	N
Guangxi/LZ21	2015	China	E	D	D	E	H	R	N
Guangxi/LZ317	2013	China	E	D	D	E	H	R	N
Guangxi/LZ36	2015	China	E	D	D	E	H	R	N
Guangxi/LZ52	2015	China	E	D	D	E	H	R	N
Guangxi/LZ56	2015	China	E	D	D	E	H	R	N
Guangxi/NN45	2014	China	E	D	D	E	H	R	N
Guangxi/NNTW15	2015	China	E	D	D	E	H	R	N
Guangxi/QZ5	2013	China	E	D	D	E	H	R	N
Guangxi/WZ1	2013	China	E	D	D	E	H	R	N
Guangxi/WZ11	2013	China	E	D	D	E	H	R	N
Guangxi/WZ2	2013	China	E	D	D	E	H	R	N
<b>Feline H1 reference</b>									
France/0514	2009	France	E	D	D	E	H	R	N
Iowa/26991	2009	USA	N/A	N/A	N/A	E	H	R	N
Oregon/29573	2009	USA	N/A	N/A	N/A	E	H	R	N
Pansylvania/30187	2009	USA	N/A	N/A	N/A	E	H	R	N

### 3.5. Discussion

Influenza A virus cause respiratory diseases in many host species worldwide. To our knowledge, this study is the first report of detection and genetic characterization of pdmH1N1-2009 in dog in Thailand. Up to date, there are only four complete genomes of pdmH1N1-2009 recovered from dogs and cats available in the GenBank database. In Thailand, the previous study based on serological survey showed that pdmH1N1-2009 was predominant subtype circulating in dogs in Thailand. Our result provided the additional evidence of genetic characteristics of the pdmH1N1-2009 in dogs. For our survey, the (n=571) nasal swab samples were collected from dogs with respiratory signs during November 2015 to December 2018. Our results showed that (1.23%) 7/571 of nasal swab samples were positive for influenza A virus. The occurrence of influenza A virus in dogs was relatively high during the winter season in Thailand (November to February) which similar finding of high occurrence of influenza in the cold season has also been reported. In this study, dogs of all ages could be infected with influenza virus, but younger dogs (<1 year) are more positive which in agreement to previous studies. The amount of co-infection of IAV-CRCoV (n=2) and IAV-CPIV-5 (n=1) were observed.

In this study, we identified subtype of influenza virus as pandemic H1N1-2009. The previous study in Thailand in 2012, canine influenza virus isolated from dogs with respiratory signs was identified as influenza A virus subtype H3N2 of avian origin. The phylogenetic analysis of H1 gene of Thai-pdmH1N1-2009 (AD105) in this study showed that the virus clustered into the pdm/2009 lineage and closely related to pdmH1N1-2009 from cats in France (France/0514). For N1 gene, Thai-pdmH1N1-2009 (AD105) was grouped into pdm/2009 lineage and closely related to swine influenza virus (CU3803) from Thailand. The phylogenetic analysis of internal genes (PB1, PB2, PA, M, Ns and NP genes), Thai-pdmH1N1-2009 (AD105) was grouped into pdm/2009 lineage and closely related pdmH1N1-2009 from humans, pigs, cats, and dogs. It is noted that the whole genome sequences of the pdmH1N1-2009 from dogs has never been reported in Thailand. Our result supported that multiple-species transmission of padH1N1-2009 have been occurred in nature due to the spill over of the viruses from human hosts (Li et al., 2010b; Lyoo et al., 2015; Na et al., 2015a; Song et al.,

2015a). The pdm H1N1 2009 in dogs and cats have been reported in China, Korea, Italy, France and USA (Campagnolo et al., 2011; Chen et al., 2018; Lin et al., 2012a). Recently, natural reassortant of human pdmH1N1-2009 and CIV-H3N2 have been reported in dogs in China and Korea suggesting the co-infection of human influenza viruses or pdmH1N1-2009 in dogs might contribute to rapid evolution of influenza virus with high virulent and infectivity (Chen et al., 2018; Na et al., 2015b; Song et al., 2012b).

For genetic analysis, It has been known that the interspecies transmission of the viruses between humans, pigs, cats and dogs could contribute to the adaptations or mutations for the fitness of viruses to new host (Borland et al., 2020; Chen et al., 2018). In this study, the HA cleavage site, receptor binding site of Thai-pdmH1N1-2009 (AD105) contained similar amino acids to human viruses, suggesting the virus can infect or replicate in mammal such as human (Connor et al., 1994; Gao et al., 2009). The amino acid substitution analysis of internal genes showed that Thai-pdmH1N1- 2009 (AD105) was correlated to more virulence and sensitive to oseltamivir which similar to pdmH1N1-2009 from dogs, cats, pigs and humans (Takashita et al., 2015).

### 3.6 Conclusion

In this thesis, one influenza A virus was successfully subtyped and characterized by whole genome sequencing. The virus was designated as pandemic H1N1-2009 influenza A virus. This study is the first report of the detection and characterization of pdmH1N1-2009 in dog in Thailand. Phylogenetic analysis of each gene segment of the canine pandemic H1N1-2009 characterized in this study showed that the Thai canine pdmH1N1-2009 was grouped with the pdmH1N1-2009 circulating in human, dogs, cats and pigs. The genetic characteristics of the virus was resembled to those of the pdmH1N1-2009. Base on result of this study we can speculate that the reverse zoonotic infection from owner or person who close contact with dog could be occurred. Currently, there is no evidence of cross-species transmission of pdmH1N1-2009 from dogs back to humans. Since companion animals are commonly close contact with human, thus the animals can be infected with influenza A virus and can serve as “a mixing vessel” to generate novel reassortants /viruses to humans. The routine monitoring of influenzas A viruses in companion animals should be further expand in large scale for better understanding of the diversity, distribution and evolution of influenza A viruses in dogs. Risk communications and practices regarding to the risk of influenza virus infection among owners, veterinarians or persons which in close contact with animals should be provided especially in high human-dog interface settings.

## CHAPTER IV

### CANINE RESPIRATORY CORONAVIRUSES (CRCoV)

The manuscript is in preparation in title

#### Genetic diversity of canine respiratory coronaviruses (CRCoV) in dogs, Thailand

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#### 4.1 Abstract

Canine respiratory coronavirus (CRCoV) is one of the pathogens causing canine infectious respiratory complex (CIRD) in dogs worldwide. During November 2015 to December 2018, we survey CRCoV infection in dogs with respiratory signs in small animal hospitals in Bangkok, Thailand. Our result showed that 13.13% (75/571) of canine samples were positive for CRCoV by using one-step RT-PCR. To characterize the virus, we selected 2 Thai-CRCoVs for whole genome sequencing and 14 Thai-CRCoVs for complete HE and S gene sequencing. Pairwise sequence comparison and phylogenetic analysis results showed that Thai-CRCoVs were grouped into betacoronavirus group A and closely related to CRCoV from dogs in South Korea. Time of most recent ancestor analysis (TMRCA) showed that Thai-CRCoVs were estimated to separate from human coronavirus (HCoV-OC43) and bovine coronavirus (BCoV) with the most recent common ancestor since 2004, suggesting that Thai-CRCoVs potential originated from interspecies transmission. between dog, human and bovine. Thus, the survey of CRCoV should be investigated on a larger scale to determine the distribution, genetic diversity, and possibly cross-species transmission of CRCoVs in the future.

**Keywords:** Genetic diversity, Coronavirus, CRCoV, Dogs, Thailand, Zoonosis



## 4.2 Introduction

Coronavirus (CoV) is an enveloped, non-segmented positive, single-stranded RNA virus of the family *Coronaviridae*. CoV can be classified to four genus, namely alphacoronavirus, betacoronavirus, deltacoronavirus and gammacoronavirus. CoVs cause respiratory and gastrointestinal diseases in several animal species including bats, pigs, cats, dogs, birds, ruminants, mice, and humans (Woo et al., 2010b). Canine respiratory coronavirus (CRCoV) belongs to the genus *betacoronavirus* and differentiate from canine enteric coronavirus in *alphacoronavirus* (CCoV).

CRCoV was first reported in dogs with severe respiratory illnesses in UK (Erles et al., 2003a). CRCoV infection in dogs have been reported worldwide including Asia (China, Japan, and South Korea), Europe (Italy, Sweden and UK) and USA (An et al., 2010; Mitchell et al., 2013). CRCoV is one of the pathogens causing canine infectious respiratory complex (CIRD) including canine influenza virus, canine parainfluenza virus type 5, canine adenovirus type 2, canine pneumovirus, *Bordetella bronchiseptica* and *Mycobacterium cynos*. Clinical signs of CRCoV infection in dogs are vary from mild to severe respiratory signs and occasional fatal due to bronchopneumonia and secondary bacterial infection (Priestnall et al., 2009). CRCoVs are highly contagious particularly in kennel dogs (Mitchell et al., 2013). Previous study showed that the prevalence of CRCoV infection in dogs was approximately 3-15% , while the seroprevalence could be higher upto 30-80% (Wille et al., 2020a)

CRCoV is closely related to human coronavirus (HCoV-OC43) and bovine coronavirus (BCoV) (Erles et al., 2007). Previous studies indicated that the viruses share common ancestor and might have ability of cross-species transmission (Vijgen et al., 2005). In 2019, novel SARCoV-2 (COVID-2019) was emerged and speculated that the virus was originated from cross-species transmission between animals to humans. Therefore, the outbreak of COVID-2019 raised the public health concern of other coronaviruses in animals (Yoo and Yoo, 2020). Since dog-human interface is common especially in high-density area or urban setting. This could elevate the risk of zoonotic transmission from dogs to human, especially among persons who have routine closed contact with animals including veterinary practitioners and animal care workers (Baker and Gray, 2009). Currently, the study of coronavirus in dogs is still

limit. There are only 3 whole genomes sequences of CRCoV available at the GenBank database. In this study, we aimed to survey CRCoVs in domestic dogs in Thailand and genetic characterize the virus by whole genome sequencing.



### 4.3. Materials and Methods

#### 4.3.1. Sample collection from dogs

During November 2015 to December 2018, we collected 571 nasal swab samples from dogs with respiratory signs including sneezing, nasal discharge, cough, and dyspnea. The nasal swab samples were collected from 12 private small animal hospitals in Bangkok, Thailand. The animal demographic data including age, sex, breed, and vaccination history were recorded. This study was conducted under the approval protocol of the Chulalongkorn University's Animal Care and Use Committee (CU-IACUC # 1731074).

#### 4.3.2. Identification of canine respiratory coronavirus (CRCoV)

All nasal swab samples (n=571) were subjected to RNA extraction by using QIAasymphony DSP viral/Pathogen mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. RNA was stored at  $-20^{\circ}\text{C}$  until use. CRCoV identification was performed by using one-step RT-PCR with RdRp gene specific primers. The primers used in this study were previously described (Lu et al., 2017). Briefly, one-step RT-PCR (Invitrogen, USA) was conducted in a final volume of 25  $\mu\text{l}$  comprising 3  $\mu\text{l}$  of template RNA, 15  $\mu\text{l}$  of 2xReaction Mix, 0.6  $\mu\text{l}$  of 10  $\mu\text{l}$  forward (CRCoV-RdRp-F; TGATATTTGCAATGCTAG) and reward primer (CRCoV-RdRp-R; CATAACCAATCCTTCTTAG), 1.2  $\mu\text{l}$  of SuperScript III RT and distilled water to final volume 25  $\mu\text{l}$ . For RT-PCR condition, the reaction contained cDNA synthesis step at  $55^{\circ}\text{C}$  for 30 minutes, next to an initial denaturation step at  $94^{\circ}\text{C}$  for 2 min, following 40 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $48^{\circ}\text{C}$  for 30 s and extension at  $68^{\circ}\text{C}$  for 30 s, as well as, final extension step at  $68^{\circ}\text{C}$  for 6 min. Then 4  $\mu\text{l}$  of PCR product was run on a 1.5% agarose gel electrophoresis. The expected amplified product size of CRCoV was 386 bp. Moreover, all of samples were screened for other important canine respiratory viruses (Canine parainfluenza virus type5 (CPiV-5) and Canine influenza virus (CIV).

#### 4.3.3. Characterization of canine respiratory coronavirus (CRCoV)

In this study, CRCoVs were subjected to either whole genome sequencing (n=2) or S, HE gene sequencing (n=14). The criteria for CRCoVs selection for genetic

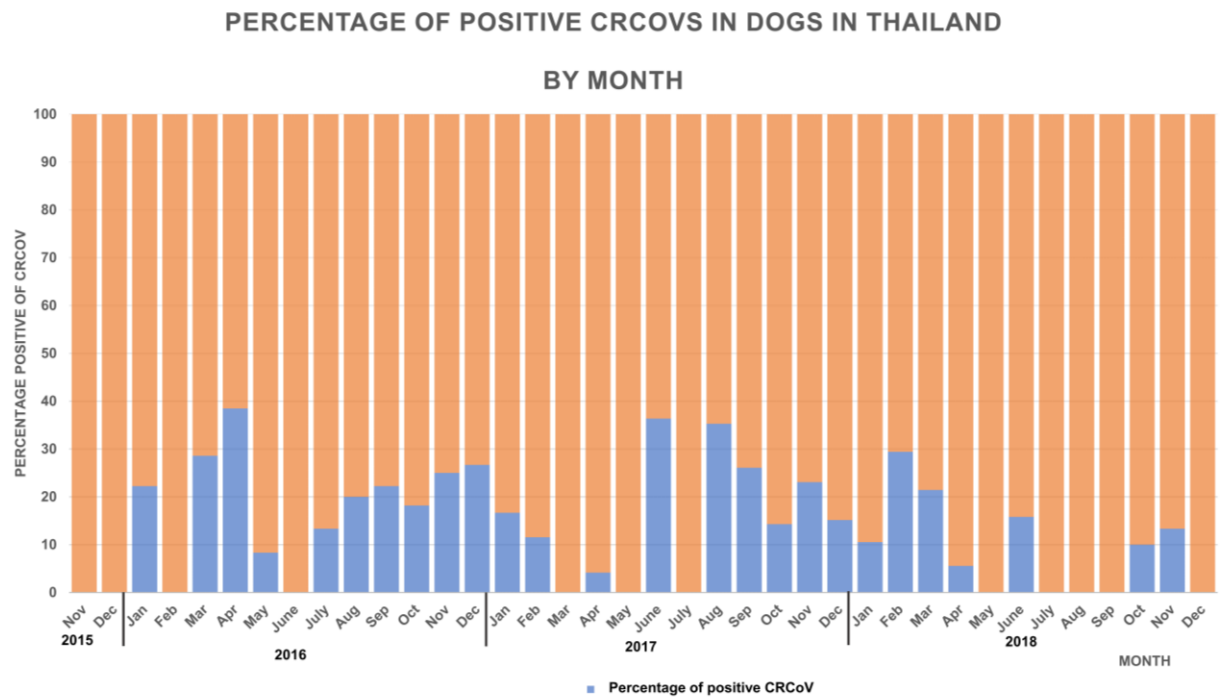
characterization were based on epidemiological and demographic information such as age of dog, date of isolation, breed, and vaccination history. For whole genome sequencing and S, HE genes sequencing, the virus genes were amplified by PCR with newly designed primers by using Primer 3 plus (Steve Rozen et al., 2000). The primers sequences are available upon requested. The nucleotide sequencing was conducted at 1st Base Laboratories Sdn Bhd, Malaysia. The nucleotide sequences were validated and assembled by SeqMan software v.5 v.5.03 (DNASTAR Inc.; Wisconsin, USA).

Phylogenetic analysis was conducted by comparing nucleotide sequences of each gene of Thai CRCoVs with those of CoVs available from the GenBank database. The reference CRCoVs were selected based on different geographic locations and host species. Phylogenetic tree was constructed by using MEGA v.7.0 (Tempe, AZ, USA) applying neighbor-joining method with Kimura 2-parameter with 1,000 bootstrap replication (Tamura et al., 2013). The evolution analysis was constructed by using BEAST 1.10 with Bayesian Markov Chain Monte Carlo (BMCMC) algorithm. A strict clock model with a coalescent population and GTR (I) substitution were used as model parameters. The Bayesian MCMC chain lengths were 10,000,000 generations, with sampling every 10,000 generations. The tree iteration was discharged with 10% of the chains as a burn-in pattern by using a tree annotator. The parameters were confirmed by calculating the Effective Sample Size (ESS) with the TRACER program v. 1.7.1 (Molecular evolution, phylogenetics and epidemiology, Edinburgh, Scotland, UK). The minimum standard error in each gene segment was analyzed by ESS values greater than 200. The resulting MCC tree was drawn with FigTree software (v1.4.2) (Molecular evolution, phylogenetics and epidemiology, Edinburgh, Scotland, UK). For genetic analysis, the nucleotide sequences and deduced amino acids of CRCoVs and reference viruses were aligned and compared by using MegAlign software v.5.03 (DNASTAR Inc.; Wisconsin, USA).

#### 4.4. Result

##### 4.4.1. Canine Respiratory Coronavirus (CRCoV) in domestic dogs

In this study, we investigated CRCoV infection in dogs with respiratory signs in small animal hospitals in Bangkok, Thailand during November 2015 to December 2018. Our result showed that 13.13% (75/571) of nasal swabs were positive to CRCoV by using one-step RT-PCR specific to RdRp gene. In detail, for three years surveillance, by month, CRCoVs positivity was highest in 2016 (November–December, 25.00–26.60 %), in 2017 (June and August; 35.29–36.36%) and in 2018 (February ;29.41%) (Figure 4.1). By age of dogs, CRCoVs were frequently detected in dogs older than 5 years (16.67%) and dogs younger than 1 year (16.25%) (Table 4.1). In this study, the samples were also examined for other important canine respiratory viruses including influenza A (CIV) and canine parainfluenza virus type 5 (CPIV-5). Co-infection of CRCoVs with CIV (n=1) and CPIV-5 (n= 5) were observed (data not showed).



**Figure 4.1.** Percentage of CRCoVs positivity in dogs in Bangkok Thailand by months during NoV 2015- Dec 2018.



**Table 4.1.** Associations between age of CRCoVs in this study.

Age	Number	CRCoVs positive (%)
Young <1 year	240	39 (16.25%)
Adult (1-5 years)	211	16 (7.58%)
Older (>5 years)	120	20 (16.67%)
	<b>571</b>	<b>75 (13.13%)</b>



#### 4.4.2. Genetic characteristics of Thai CRCoV

In this study, two CRCoVs (AD-21 and AD-431) were subjected to whole genome sequencing and other CRCoVs (n=14) were selected and subjected to S and HE gene sequencing. The nucleotide sequences of Thai CRCoVs characterized in this study were available in the GenBank database under the accession numbers on Table 4.2 and Table 4.3. Analysis of whole genome sequences of Thai CRCoVs (AD-21 and AD-431) revealed that the viruses possessed highest nucleotide similarities to CRCoV from China (BJ232) (99.80% nucleotide identities, 93.60% amino acid identities) (Table 4.4). Thai CRCoV also possessed high nucleotide similarity to bovine coronavirus (BoV; Kakegawa) (98.30% nucleotide identities, 90.90% amino acid identities) and Human coronavirus (HCoV-OC43; ATCC VR-759) (95.80% nucleotide identities, 87.00% amino acid identities). Phylogenetic analysis based on whole genome sequences showed that Thai CRCoVs (AD-21 and AD-431) belong to betacoronavirus group A which including BoV, HCoV-OC43 and HeCoV-4408. On the other hand, Thai CRCoVs were clustered in separated clusters from canine enteric coronavirus (CoV) of alphacoronavirus group (40.90-41.10% nucleotide identities) and canine SARS-CoV2 of betacoronavirus group (49.80% nucleotide identities) (Figure 4.2 and Table 4.4).



**Table 4.2.** Description of canine respiratory coronaviruses detected in this study.

No.	Virus ID	Date of isolation	Age	Breed	Sequencing	GenBank accession number
1	AD 21	Jan-16	4 Months	Mixed	WGS	N/A
2	AD 22	Jan-16	4 Months	Mixed	N/A	N/A
3	AD 113	Mar-16	3 Months	Pomeranian	N/A	N/A
4	AD 126	Mar-16	3 Months	Shih-Tzu	N/A	N/A
5	AD 129	Apr-16	3 Months	Siberian Husky	PGS (S, HE)	N/A
6	AD 130	Apr-16	9 Months	Shih-Tzu	N/A	N/A
7	AD 138	Apr-16	2 Years	Mixed	N/A	N/A
8	AD 143	Apr-16	4 Months	Pomeranian	N/A	N/A
9	AD144	Apr-16	5 Years	Golden Retriever	N/A	N/A
10	AD 149	May-16	10 Years	Dalmatian	N/A	N/A
11	AD 242	Jul-16	6 Years	Pomeranian	PGS (S, HE)	N/A
12	AD 260	Jul-16	3 Months	Bully	N/A	N/A
13	AD 312	Aug-16	9 Years	Shih-Tzu	N/A	N/A
14	AD 319	Aug-16	9 Years	Shih-Tzu	N/A	N/A
15	AD 331	Sep-16	3 Months	Pomeranian	N/A	N/A
16	AD 339	Sep-16	3 Months	Beagle	N/A	N/A
17	AD 357	Oct-16	3 Months	Pomeranian	N/A	N/A
18	AD 363	Oct-16	2 Months	Pomeranian	N/A	N/A
19	AD 368	Nov-16	3 Years	Labrador Retriever	N/A	N/A
20	AD 373	Nov-16	3 Months	Pomeranian	N/A	N/A
21	AD 374	Nov-16	2 Years	Pug	N/A	N/A
22	AD 381	Dec-16	3 Months	Pekingese	N/A	N/A
23	AD 385	Dec-16	3 Years	Mixed	N/A	N/A
24	AD 386	Dec-16	3 Years	Mixed	PGS (S, HE)	N/A
25	AD 388	Dec-16	1 Year	Mixed	N/A	N/A
26	AD 404	Jan-17	5 Months	Thai Bangkaew	PGS (S, HE)	N/A
27	AD 406	Jan-17	3 Months	Pomeranian	N/A	N/A
28	AD 431	Feb-17	4 Months	Mixed	WGS	N/A
29	AD 435	Feb-17	5 Months	Mixed	PGS (S, HE)	N/A
30	AD 436	Feb-17	1 Year	Mixed	PGS (S, HE)	N/A
31	AD518	Apr-17	2 Months	Beagle	PGS (S, HE)	N/A
32	AD541	Jun-17	2 Months	Shih-Tzu	N/A	N/A
33	AD557	Jul-17	2 Months	Beagle	PGS (S, HE)	N/A
34	AD558	Jul-17	3 Years	Pomeranian	N/A	N/A
35	AD563	Jul-17	4 Months	Pomeranian	N/A	N/A
36	AD564	Aug-17	>5 Years	Golden Retriever	PGS (S, HE)	N/A
37	AD565	Aug-17	>5 Years	Bully	N/A	N/A
38	AD566	Aug-17	>5 Years	Thai Bangkaew	N/A	N/A
39	AD567	Aug-17	>5 Years	Pomeranian	N/A	N/A
40	AD572	Aug-17	5 Months	Mixed	N/A	N/A
41	AD579	Aug-17	3 Years	Yorkshire Terrier	N/A	N/A
42	AD584	Sep-17	7Years	Labrador Retriever	PGS (S, HE)	N/A
43	AD586	Sep-17	3 Months	Mixed	N/A	N/A
44	AD587	Sep-17	3 Months	French bulldog	N/A	N/A
45	AD588	Sep-17	> 5 Years	Labrador Retriever	N/A	N/A
46	AD 591	Sep-17	> 5 Years	Chihuahua	N/A	N/A

**Table 4.2.** Description of canine respiratory coronaviruses detected in this study.  
(cont.)

No.	Virus ID	Date of isolation	Age	Breed	Sequencing	GenBank accession number
47	AD 594	Sep-17	>5 Years	Beagle	N/A	N/A
48	AD 2016	Oct-17	> 5 Years	Thai Bangkaew	N/A	N/A
49	AD 20105	Oct-17	6 Months	Pug	N/A	N/A
50	AD 20109	Oct-17	>5 Years	Chihuahua	N/A	N/A
51	AD 20149	Nov-17	>5 Years	Thai Bangkaew	N/A	N/A
52	AD 20169	Nov-17	>5 Years	Cocker Spaniel	N/A	N/A
53	AD 20232	Nov-17	>5 Years	Mixed	N/A	N/A
54	AD 20250	Dec-17	2 Months	Pomeranian	PGS (S, HE)	N/A
55	AD 20272	Dec-17	4 Years	Mixed	N/A	N/A
56	AD 20273	Dec-17	2 Months	Pomeranian	N/A	N/A
57	AD 20356	Dec-17	3 Months	Mixed	N/A	N/A
58	AD 20364	Dec-17	3 Months	Pomeranian	N/A	N/A
59	AD20379	Jan-18	4 Years	Yorkshire Terrier	N/A	N/A
60	AD 20407	Jan-18	2 Years	Mixed	N/A	N/A
61	AD 20801	Feb-18	3Years	Mixed	N/A	N/A
62	AD 20803	Feb-18	3 Years	Mixed	N/A	N/A
63	AD 20804	Feb-18	5 Years	Mixed	N/A	N/A
64	AD 20805	Feb-18	10 Years	Pomeranian	N/A	N/A
65	AD 20806	Feb-18	3 Months	Pomeranian	PGS (S, HE)	N/A
66	AD 20905	Mar-18	5 Months	Pomeranian	N/A	N/A
67	AD 20912	Mar-18	2 Months	Pug	N/A	N/A
68	AD 20913	Mar-18	3 Months	Mixed	N/A	N/A
69	AD 21269	Apr-18	4 Months	Mixed	PGS (S, HE)	N/A
70	AD 21756	Jun-18	3 Months	Pomeranian	N/A	N/A
71	AD 21797	Jun-18	3 Months	Yorkshire Terrier	PGS (S, HE)	N/A
72	AD 21798	Jun-18	1 Year	Chihuahua	N/A	N/A
73	AD 22478	Oct-18	3 Years	Mixed	N/A	N/A
74	AD 22496	Nov-18	5 Months	Pomeranian	N/A	N/A
75	AD 22747	Nov-18	2 Months	Siberian Husky	N/A	N/A

**Table 4.3.** Detail description of canine respiratory coronaviruses (CRCoVs) characterized this study.

Virus ID	Date of isolation	Age	Breed	Sequencing	Coronavirus Group
CRCoV					
AD 21	Jan-16	4 Months	Mixed	WGS	Betacoronavirus group
AD 431	Feb-17	4 Months	Mixed	WGS	Betacoronavirus group
AD 129	Apr-16	3 Months	Siberian Husky	PGS (S, HE)	Betacoronavirus group
AD 242	Jul-16	6 Years	Pomeranian	PGS (S, HE)	Betacoronavirus group
AD 386	Dec-16	3 Years	Mixed	PGS (S, HE)	Betacoronavirus group
AD 404	Jan-17	5 Months	Thai Bangkaew	PGS (S, HE)	Betacoronavirus group
AD 435	Feb-17	5 Months	Mixed	PGS (S, HE)	Betacoronavirus group
AD 436	Feb-17	1 Year	Mixed	PGS (S, HE)	Betacoronavirus group
AD518	Apr-17	2 Months	Beagle	PGS (S, HE)	Betacoronavirus group
AD557	Jul-17	2 Months	Beagle	PGS (S, HE)	Betacoronavirus group
AD564	Aug-17	5 Years	Golden Retriever	PGS (S, HE)	Betacoronavirus group
AD584	Sep-17	7Years	Labrador Retriever	PGS (S, HE)	Betacoronavirus group
AD 20250	Dec-17	2 Months	Pomeranian	PGS (S, HE)	Betacoronavirus group
AD 20806	Feb-18	3 Months	Pomeranian	PGS (S, HE)	Betacoronavirus group
AD 21269	Apr-18	4 Months	Mixed	PGS (S, HE)	Betacoronavirus group
AD 21797	Jun-18	3 Months	Yorkshire Terrier	PGS (S, HE)	Betacoronavirus group

\*Classification of coronavirus base on S gene sequence analysis.

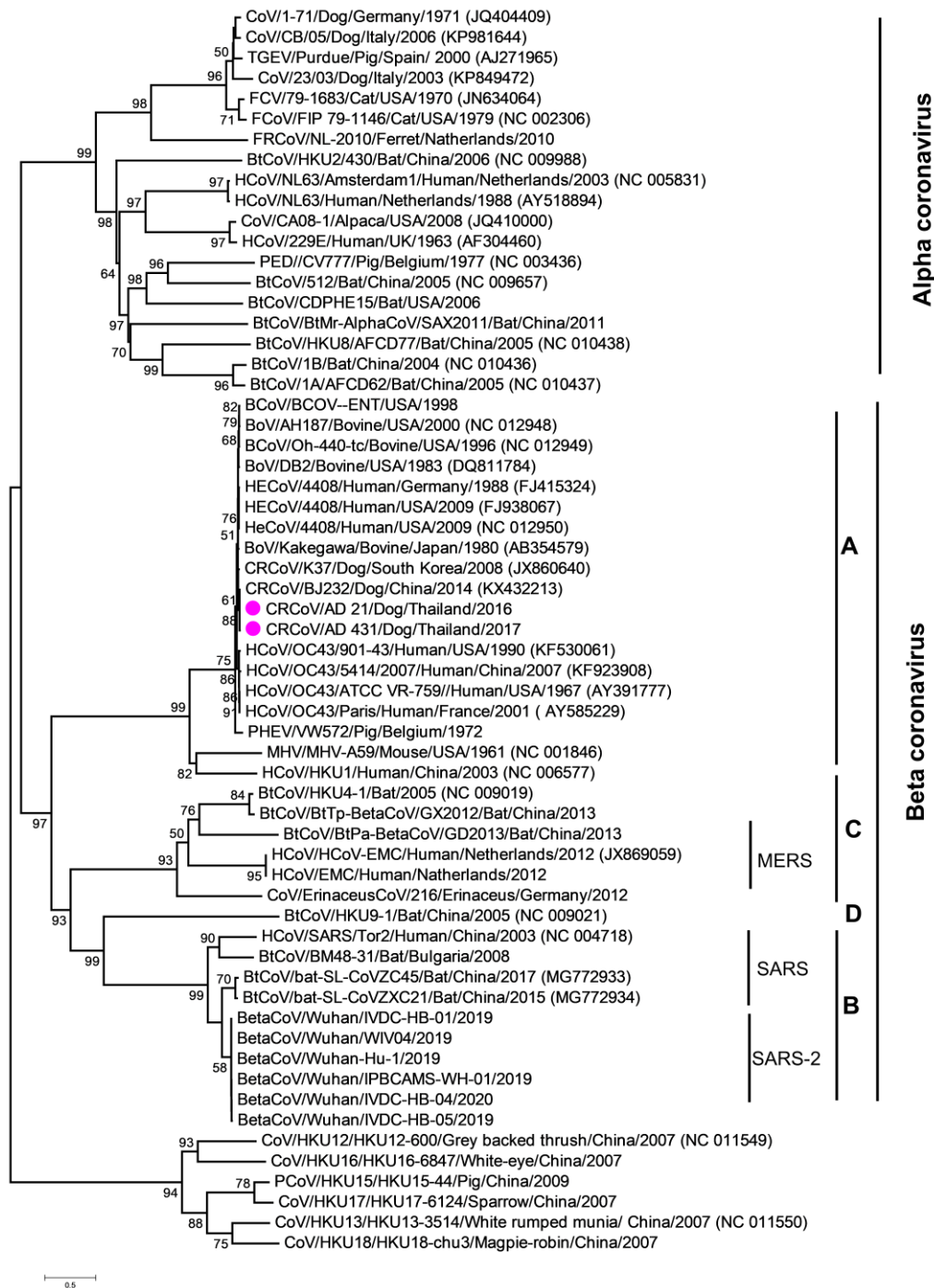
**Table 4.4.** Pair-wise sequence comparison of whole genome sequences of Thai-CRCoV and reference betacoronaviruses (CRCoV, BCoV, HCoV-OC43, HCoV-4408, SARS-CoV, SARS-CoV-2, and MERS) and alphacoronaviruses (CCoV, FCoV, HCoV-229E, PED, and TGEV) coronavirus from the GenBank database.

Virus	Accession #	Host	Country	Year	Nucleotide similarities (%)					
					WGS	ORF1ab	HE	NSP	S	M
					211-13341,13341-21494	22352-23626	21504-22340	23641-27732	28691-29383	
<b>CRCoV this study</b>										
AD21		Dog	Thailand	2016	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)
AD 431		Dog	Thailand	2017	99.90 (93.60)	99.90 (100.00)	99.10 (98.60)	100.00 (100.00)	99.80 (99.60)	99.80 (100.00)
AD 129		Dog	Thailand	2016	N/A	N/A	99.80 (99.30)	N/A	99.70 (99.60)	N/A
AD 242		Dog	Thailand	2016	N/A	N/A	99.80 (99.50)	N/A	99.70 (99.60)	N/A
AD 386		Dog	Thailand	2016	N/A	N/A	99.80 (99.50)	N/A	99.70 (99.30)	N/A
AD 404		Dog	Thailand	2017	N/A	N/A	99.80 (99.30)	N/A	99.80 (99.60)	N/A
AD 435		Dog	Thailand	2017	N/A	N/A	99.10 (98.60)	N/A	99.80 (99.60)	N/A
AD 436		Dog	Thailand	2017	N/A	N/A	99.10 (98.60)	N/A	99.80 (99.70)	N/A
AD 518		Dog	Thailand	2017	N/A	N/A	99.80 (99.50)	N/A	99.70 (99.60)	N/A
AD 557		Dog	Thailand	2017	N/A	N/A	99.80 (99.30)	N/A	99.80 (99.60)	N/A
AD 564		Dog	Thailand	2017	N/A	N/A	99.70 (99.10)	N/A	99.80 (99.60)	N/A
AD 584		Dog	Thailand	2017	N/A	N/A	99.70 (99.10)	N/A	99.80 (99.70)	N/A
AD 20250		Dog	Thailand	2017	N/A	N/A	99.70 (99.30)	N/A	99.70 (99.70)	N/A
AD 20806		Dog	Thailand	2018	N/A	N/A	99.70 (99.10)	N/A	99.70 (99.60)	N/A
AD 21269		Dog	Thailand	2018	N/A	N/A	99.80 (99.50)	N/A	99.70 (99.50)	N/A
AD 21797		Dog	Thailand	2018	N/A	N/A	99.70 (99.30)	N/A	99.80 (99.70)	N/A
<b>Reference betacoronaviruses</b>										
<b>CRCoV</b>										
BJ232	KX432213	Dog	china	2014	99.80 (93.60)	99.90 (100.00)	99.90 (99.500)	99.80 (99.60)	99.90 (99.90)	99.80 (100.00)
K37	JX860640	Dog	South Korea	2008	98.70 (91.40)	98.70 (91.50)	97.30 (96.00)	99.00 (97.70)	99.50 (99.30)	97.40 (97.70)
K39	EU983107	Dog	South Korea	2008	N/A	N/A	98.40 (96.70)	N/A	99.50 (99.20)	N/A
02/005	AB242262	Dog	Japan	2002	N/A	N/A	N/A	N/A	99.20 (99.00)	N/A
06/075	AB570269	Dog	Japan	2006	N/A	N/A	N/A	N/A	99.00 (98.80)	N/A
K9	GQ918141	Dog	South Korea	2008	N/A	N/A	98.00 (96.70)	N/A	99.50 (99.10)	N/A
240/05	EU999934	Dog	Italy	2005	N/A	N/A	98.40 (98.10)	N/A	98.90 (98.80)	N/A
4182	DQ682406	Dog	UK	2003	N/A	N/A	98.10 (97.60)	N/A	98.90 (98.10)	N/A
T101	AY150272	Dog	UK	2003	N/A	N/A	N/A	N/A	98.80 (98.00)	N/A
<b>BCoV</b>										
BCoV-ENT		Bovine	USA	1998	97.90 (90.00)	98.30 (90.90)	97.20 (95.80)	98.10 (97.30)	96.30 (95.40)	97.00 (97.30)
OH-440-tc	NC 012949	Bovine	USA	1996	98.00 (90.20)	98.40 (91.00)	96.80 (95.10)	98.30 (98.00)	96.70 (95.40)	97.20 (97.70)
DB2	DQ811784	Bovine	USA	1985	98.10 (90.50)	98.50 (91.20)	97.50 (96.20)	98.70 (97.70)	96.90 (95.90)	97.40 (97.70)
AH187	NC 012948	Bovine	USA	2000	97.90 (90.00)	98.30 (90.90)	97.00 (95.80)	98.30 (98.00)	96.50 (95.50)	97.40 (97.70)
<del>Kelesova</del>	AB334579	Bovine	Japan	1980	98.30 (90.90)	98.70 (91.70)	97.30 (96.00)	99.10 (98.00)	97.10 (95.80)	97.60 (98.20)
<b>HCoV-OC43</b>										
5414	KF923908	Human	China	2007	95.20 (86.10)	97.10 (88.60)	94.10 (89.40)	95.30 (94.90)	90.60 (87.20)	94.50 (95.00)
Paris	AY585229	Human	France	2001	95.80 (87.00)	97.40 (89.10)	95.40 (92.00)	95.70 (94.90)	93.10 (90.30)	95.50 (96.40)
901-43	KF530061	Human	USA	1990	95.70 (86.80)	97.20 (88.80)	94.90 (90.10)	95.30 (94.50)	93.30 (91.30)	95.50 (95.90)
ATCC VR-759	AY391777	Human	USA	1967	95.80 (87.00)	97.40 (89.10)	95.40 (92.00)	95.60 (94.90)	92.80 (89.80)	95.30 (96.40)
<b>HECoV-4408</b>										
<del>HECoV/4408/</del>	FJ415324	Human	Germany	1988	98.10 (90.40)	98.50 (91.20)	98.20 (96.00)	98.60 (96.90)	96.90 (95.50)	97.00 (97.30)
<del>HECoV/4408</del>	NC 012950	Human	USA	2009	98.00 (90.30)	98.40 (91.00)	98.00 (96.00)	98.30 (97.70)	96.90 (95.60)	96.30 (97.70)
<b>SARS-CoV</b>										
Tor2	NC 004718	Human	China	2003	48.30 (26.90)	52.40 (30.40)	60.10 (50.40)	38.40 (19.70)	47.30 (37.50)	34.50 (26.70)
<b>SARS-CoV2</b>										
NY/040420	MT365033	Tiger	USA	2020	49.80 (27.40)	53.50 (30.50)	61.80 (53.70)	42.30 (21.70)	47.40 (36.60)	35.70 (30.80)
Wuhan-Hu-1		Human	China	2019	49.80 (27.40)	53.50 (30.50)	62.10 (53.80)	42.30 (21.70)	47.40 (36.60)	35.70 (30.80)
20-05695	MT270814	Dog	Hong Kong	2020	49.80 (27.40)	53.50 (30.50)	62.00 (53.80)	42.30 (21.70)	47.40 (36.60)	35.70 (30.80)
<b>MERS</b>										
<del>HECoV-EMC</del>	JX869059	Human	Netherlands	2012	46.50 (26.20)	51.10 (30.30)	63.20 (52.80)	37.80 (18.30)	46.10 (35.50)	31.00 (23.10)
<b>Reference alphacoronavirus</b>										
<b>CoV</b>										
1-71	JQ404409	Dog	Germany	1971	41.10 (21.00)	45.70 (24.80)	59.90 (50.30)	27.90 (11.30)	30.90 (15.80)	29.40 (31.20)

**Table 4.4.** Pair-wise sequence comparison of whole genome sequences of Thai-CRCoV and reference betaoronaviruses (CRCoV, BCoV, HCoV-OC43, HCoV-4408, SARS-CoV, SARS-CoV-2, and MERS) and alphacoronaviruses (CCoV, FCoV, HCoV-229E, PED, and TGEV) coronavirus from the GenBank database. (cont.)

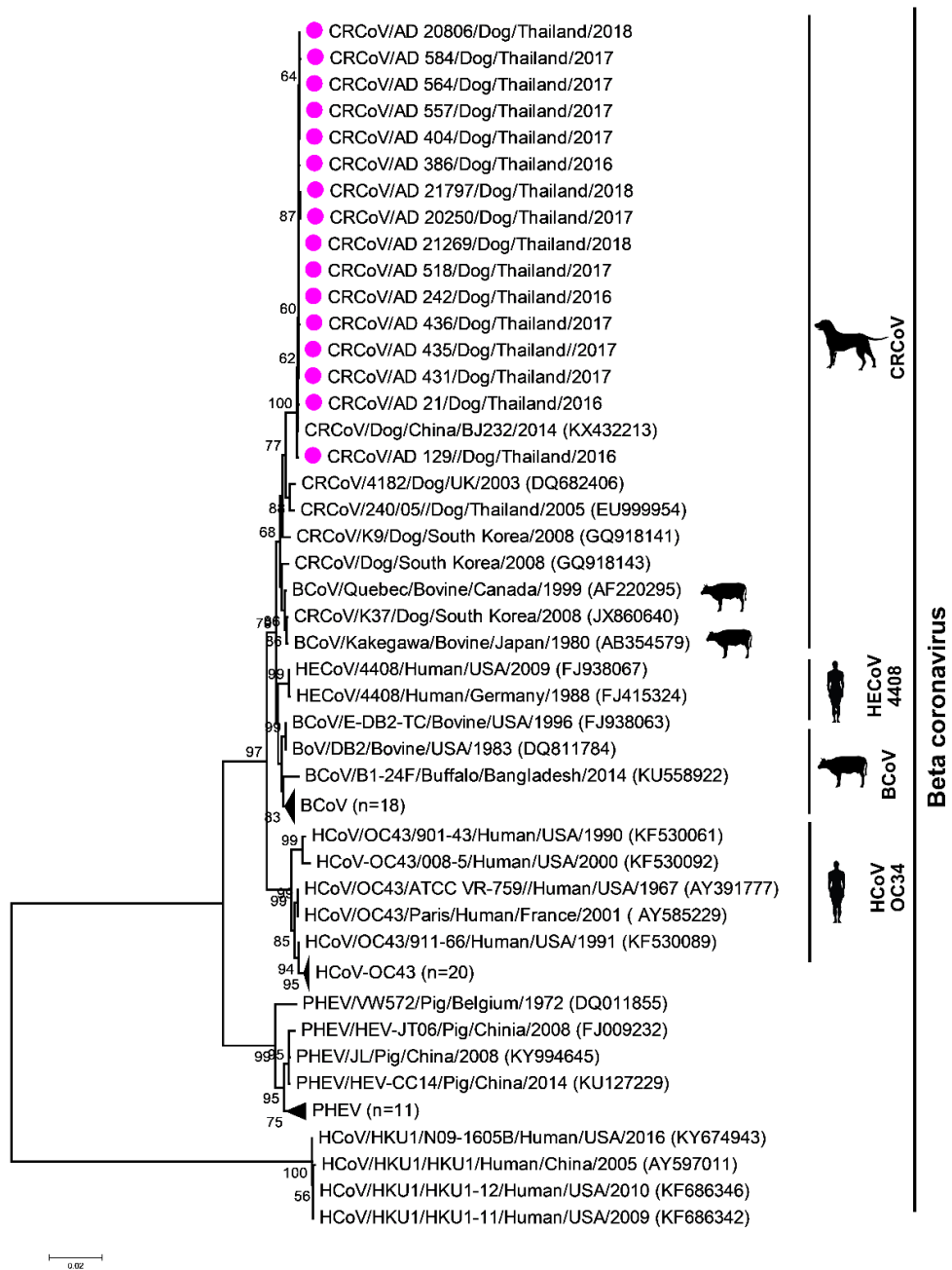
Virus	Accession #	Host	Country	Year	Nucleotide similarities (%)					
					WGS	ORF1ab 211-13341,13341-21494	HE 22352-23626	NSP 21504-22340	S 23641-27732	M 28691-29383
23/05	KPB49472	Dog	Italy	2005	41.10 (20.80)	45.50 (24.70)	60.70 (50.80)	23.70 (4.90)	27.90 (12.70)	29.20 (31.20)
CB/05	KP981644	Dog	Italy	2006	40.90 (21.00)	45.50 (24.50)	60.30 (51.10)	29.10 (11.30)	31.10 (16.50)	30.00 (31.20)
<del>FCoV</del>										
FIP 79-1146	NC 002306	cat	USA	1979	40.90 (20.80)	45.40 (24.30)	60.30 (49.90)	28.60 (11.00)	31.20 (16.80)	30.20 (31.70)
79-1683	JN634064	cat	USA	1970	40.90 (21.20)	45.50 (24.80)	60.20 (50.00)	28.30 (10.70)	31.00 (16.20)	29.00 (30.30)
HCoV-229E										
<del>HCoV</del>										
HCoV/229E	AF304460	Human	UK	1965	40.20 (21.40)	45.50 (24.50)	62.30 (52.30)	19.70 (16.30)	38.00 (28.00)	28.00 (32.10)
PED										
CV777	NC 003436	Pig	Belgium	1977	39.50 (20.90)	44.20 (23.90)	61.00 (51.70)	29.40 (15.30)	27.60 (13.60)	26.20 (33.50)
TGEV										
Purdue	AJ271965	Pig	Spain	2000	40.90 (20.90)	45.60 (24.50)	60.40 (49.90)	27.40 (8.00)	30.80 (16.30)	29.20 (31.70)





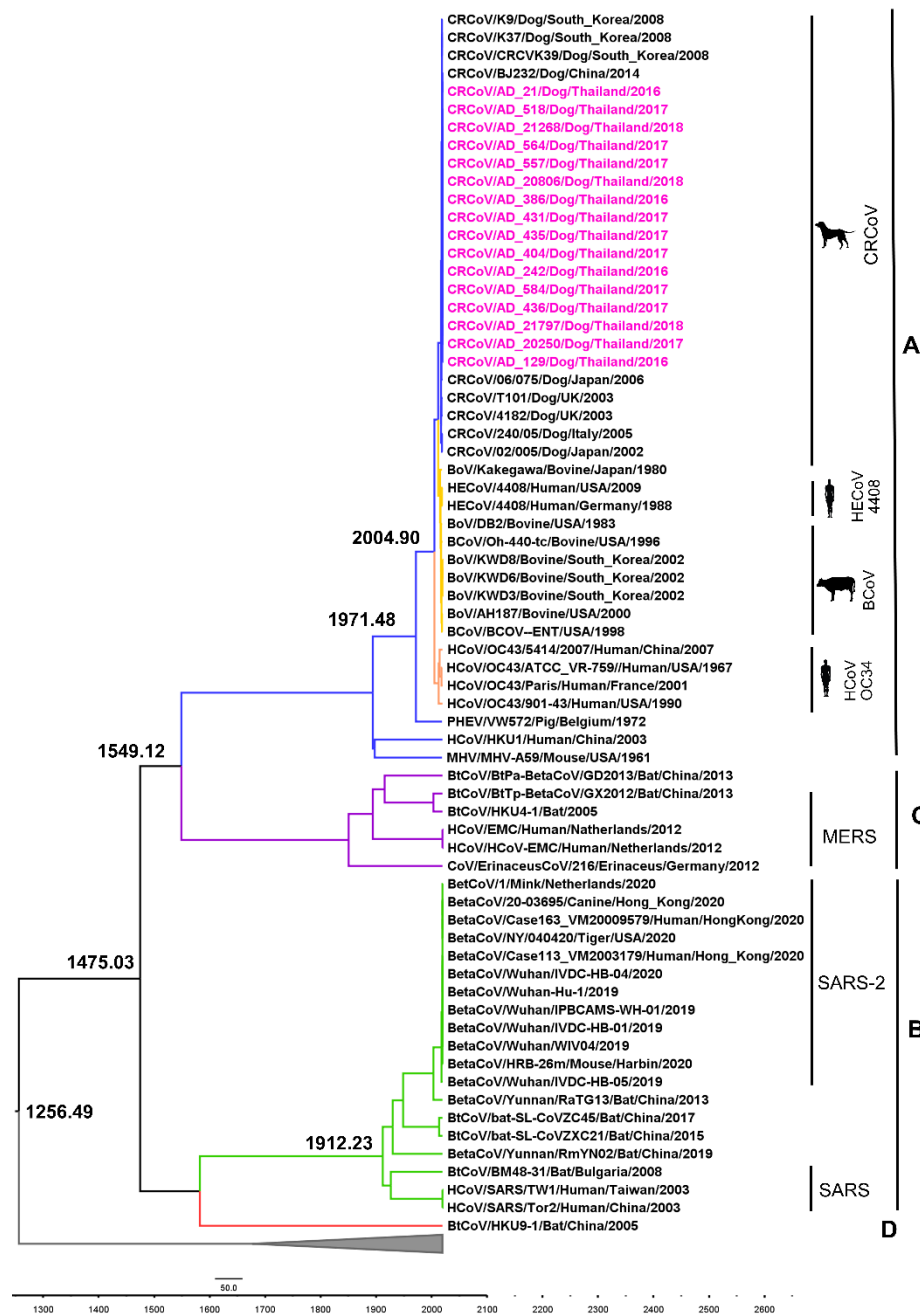
**Figure 4.2.** Phylogenetic tree based on whole genome sequence of Thai-CRCoVs and reference alphacoronaviruses and betacoronaviruses. The phylogenetic tree was constructed by using MEGA v.7.0 with neighbor-joining algorithm with kimura-2 parameter model and bootstrap analysis of 1,000 replications. Pink circle indicates Thai CRCoV in this study.

For HE gene analysis, the nucleotide identities among Thai CRCoVs were ranging from 99.10-99.80% (98.60-99.50% amino acid identities). Thai CRCoVs possessed high nucleotide similarities to CRCoV from China (BJ232) (99.90% nucleotide identities, 99.50 % amino acid identities). Thai CRCoVs also were closely related to BoV from USA (ENT) (97.20% nucleotide identities) and HCoV-OC43 (ATCC VR-759) (95.40% nucleotide identities). Phylogenetic tree of HE gene showed that Thai CRCoVs were closely related to Chinese CRCoV (BJ232) and grouped with HCoV-OC43 (901-43) and BoV (Kakegzwa) (Figure 4.3). For S gene analysis, the nucleotide identities among Thai CRCoVs were 99.70-99.80 % (99.30-99.70% amino acid identities). Thai CRCoVs possessed high nucleotide with similarities to Chinese CRCoV (BJ232) (99.90% nucleotide identities), BoV (Kakegzwa) (97.10% nucleotide identities) and HCoV-OC43 (901-43) (93.30% nucleotide identities). Furthermore, Thai CRCoVs possessed low nucleotide similarity with CoV of alphacoronavirus group (27.90-31.10% nucleotide identities). The MCC tree based on S gene, betacoronavirus can be divided into 4 groups (A-D). The Thai CRCoV were clustered with other CRCoVs as well as HCoV-4408, HCoV-OC43 and BCoV in betacoronavirus group A. While group B contains with SARS-CoV and SARS-CoV-2. Group C and D contains MERS and bat coronavirus HKU-9. It is noted that all Thai CRCoVs were clustered with other CRCoVs in betacoronavirus group A. It should be noted that TMRCA analysis base on S gene showed that Thai CRCoVs were estimated to separate from BoV and HCoV since 2004. The estimated nucleotide substitution rate of S gene was  $7.8818 \times 10^{-5}$  substitution per site per year (95% posterior densities (HPD)  $2.3042 \times 10^{-5}$ - $1.3619 \times 10^{-4}$ ) (Figure 4.4 and 4.5).

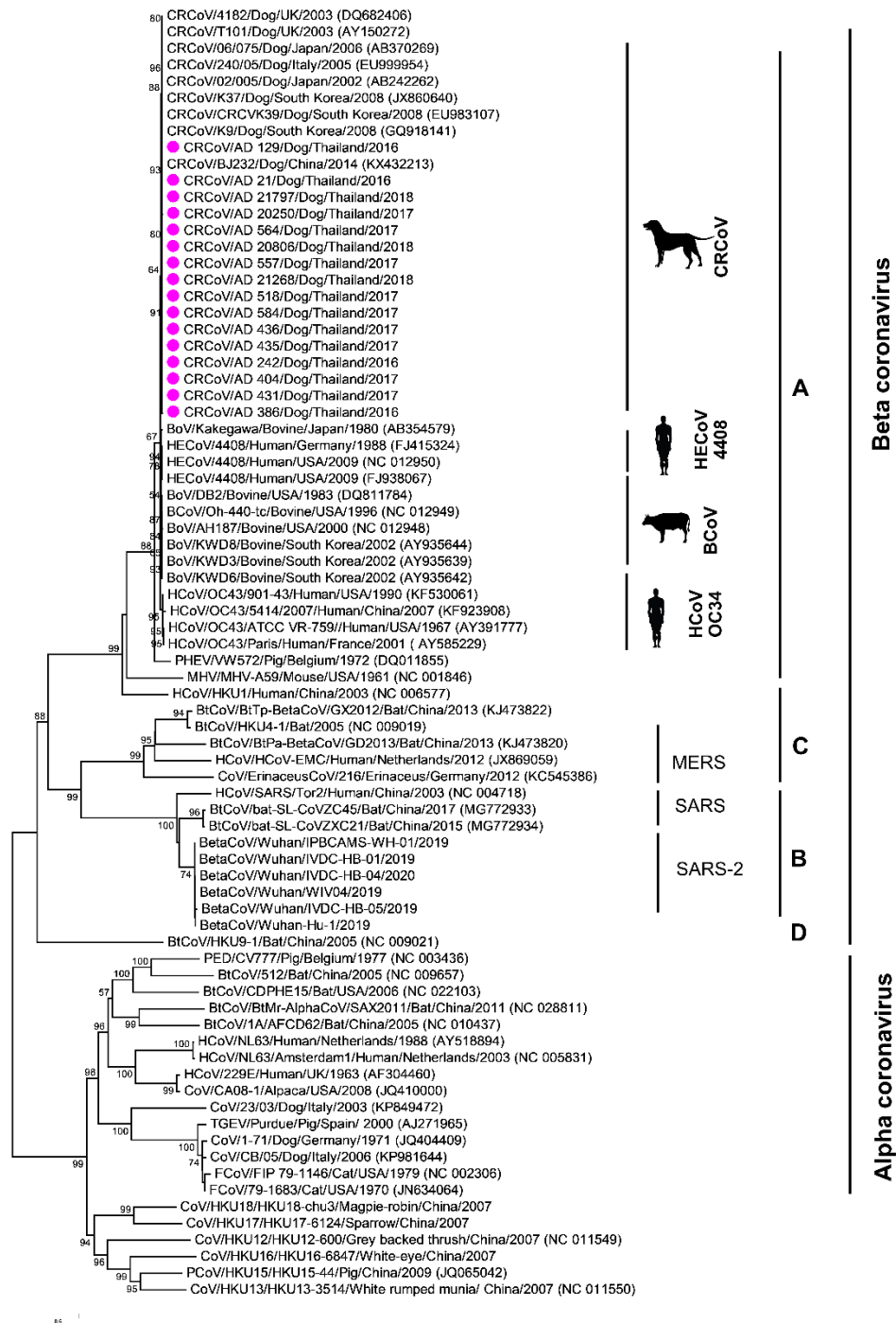


**Figure 4.3.** Phylogenetic tree based on HE gene of Thai-CRCoVs and reference alphacoronaviruses and betacoronaviruses. The phylogenetic tree was constructed by using MEGA v.7.0 with neighbor-joining algorithm with kimura-2 parameter model and bootstrap analysis of 1,000 replications. Pink circle indicates Thai CRCoV in this study.





**Figure 4.4.** The maximum clade credibility (MCC) tree base on S gene of Thai-CRCoVs and reference alphacoronaviruses and betacoronaviruses. The phylogenetic tree was constructed by using BEAST 1.10 with Bayesian Markov Chain Monte Carlo (BMCMC) algorithm. A strict clock model with a coalescent population and GTR (I) substitution were used as model parameters. Pink text indicated Thai-CRCoVs characterized in this study.

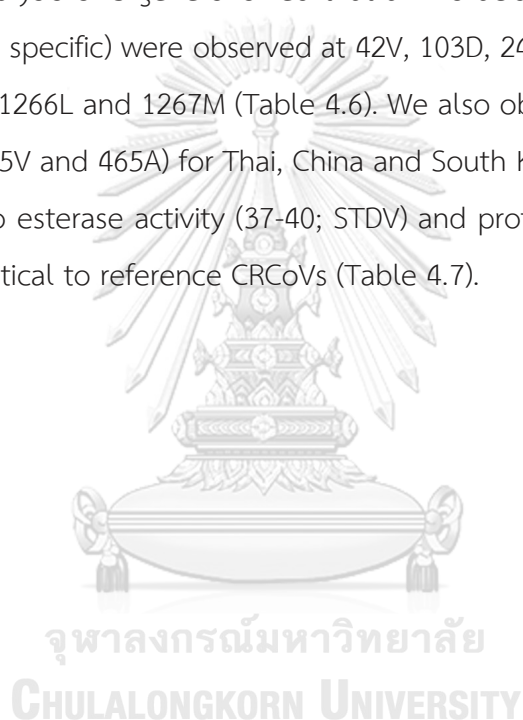


**Figure 4.5.** Phylogenetic tree based on S gene of Thai-CRCoVs and reference alphacoronaviruses and betacoronaviruses. The phylogenetic tree was constructed by using MEGA v.7.0 with neighbor-joining algorithm with kimura-2 parameter model with bootstrap analysis of 1,000 replications. Pink circle indicates Thai-CRCoV in this study.

#### 4.4.3. Genetic analysis of Thai CRCoVs

Genetic analysis of HE gene at amino acids residues related to cleavage site (18-19) and N-glycosylation (241) showed that Thai CRCoVs contained 18G, 19F and 241I which identical to other reference CRCoVs (Table 4.5). In this study, we observed unique amino acids of Thai CRCoVs and China CRCoVs at position 309L, 383H, 396A and 411S. Moreover, some Thai CRCoVs (AD431, AD 435 and AD436) have unique 4 amino acid deletions at positions 416-419 (DNGL) in HE gene.

Genetic analysis of S gene showed that amino acids residues related to host preference (canine specific) were observed at 42V, 103D, 241H, 105V, 405I, 457I, 592K, 838V, 897F, 943D, 1266L and 1267M (Table 4.6). We also observed unique amino acid sequence (28N, 225V and 465A) for Thai, China and South Korea CRCoVs. Amino acids residues related to esterase activity (37-40; STDV) and proteolytic cleavage (733-778; KRRSRR) were identical to reference CRCoVs (Table 4.7).



**Table 4.5.** Genetic analysis of HE gene of Thai-CRCoVs and reference etacoronaviruses (CRCoV, HCoV-OC43, BCoV, HCoV-4408).

Virus	Host	Country	Year	HE (1275 nt)						
				Thailand-China CRCoV specific regions				416-419 DNGI	N-glycosylation 241	Cleavage 18-19
				P309L	P383H	G396A	L411S			
<b>CRCoV in this study</b>										
AD21	Dog	Thailand	2016	L	H	A	S	DNGI	I	GF
AD 431	Dog	Thailand	2017	L	H	A	S	Deletion	I	GF
AD 129	Dog	Thailand	2016	L	H	A	S	DNGI	I	GF
AD 242	Dog	Thailand	2016	L	H	A	S	DNGI	I	GF
AD 386	Dog	Thailand	2016	L	H	A	S	DNGI	I	GF
AD 404	Dog	Thailand	2017	L	H	A	S	DNGI	I	GF
AD 435	Dog	Thailand	2017	L	H	A	S	Deletion	I	GF
AD 436	Dog	Thailand	2017	L	H	A	S	Deletion	I	GF
AD 518	Dog	Thailand	2017	L	H	A	S	DNGI	I	GF
AD 557	Dog	Thailand	2017	L	H	A	S	DNGI	I	GF
AD 564	Dog	Thailand	2017	L	H	A	S	DNGI	I	GF
AD 584	Dog	Thailand	2017	L	H	A	S	DNGI	I	GF
AD 20250	Dog	Thailand	2017	L	H	A	S	DNGI	I	GF
AD 20806	Dog	Thailand	2018	L	H	A	S	DNGI	I	GF
AD 21269	Dog	Thailand	2018	L	H	A	S	DNGI	I	GF
AD 21797	Dog	Thailand	2018	L	H	A	S	DNGI	I	GF
<b>Reference CRCoV</b>										
BJ232	Dog	china	2014	L	H	A	S	DNGI	I	GF
K37	Dog	South Korea	2008	P	P	G	L	DNGT	I	GF
K9	Dog	South Korea	2008	P	P	G	L	DNGT	I	GF
240/05	Dog	Italy	2005	P	P	G	L	DNGI	I	GF
4182	Dog	UK	2003	P	P	G	L	DNGI	I	GF
<b>Bovine coronavirus</b>										
BCOV-ENT	Bovine	USA	1998	P	P	G	L	DNGT	I	GF
Oh-440-tc	Bovine	USA	1996	P	P	G	L	DNGT	I	GF
DB2	Bovine	USA	1983	P	P	G	L	DNGT	I	GF
AH187	Bovine	USA	2000	P	P	G	L	DNGT	I	GF
Kakegawa	Bovine	Japan	1980	P	P	G	L	DNGT	I	GF
<b>HCoV-OC43</b>										
Paris	Human	France	2001	P	P	G	L	DNVT	I	GF
901-43	Human	USA	1990	P	P	G	L	DNVT	I	GF
ATCC VR-759	Human	USA	1967	P	P	G	L	DNVT	I	GF
<b>HECoV-4408</b>										
HECoV/4408	Human	Germany	1988	P	P	G	L	DNGT	I	GF
HECoV/4408	Human	USA	2009	P	P	G	L	DNGT	I	GF

**Table 4.6.** Genetic analysis of S gene of Thai-CRCoVs and reference betacoronaviruses (CRCoV, BoV, HCoV-OC43 and HCoV-4408).

Viruses	Host	Country	Year	Canine species-specific region of S gene *											
				T42V	N103D	N241H	I105V	V450I	T457I	Q592K	A838V	V897F	E943D	S1266L	V1267M
<b>CRCoV in this study</b>															
AD21	Dog	Thailand	2016	V	D	N	V	I	I	K	V	F	D	L	M
AD 431	Dog	Thailand	2017	V	D	N	V	I	I	K	V	F	D	L	M
AD 129	Dog	Thailand	2016	V	D	N	V	I	I	K	V	F	D	L	M
AD 242	Dog	Thailand	2016	V	D	N	V	I	I	K	V	F	D	L	M
AD 386	Dog	Thailand	2016	V	D	N	V	I	I	K	V	F	D	L	M
AD 404	Dog	Thailand	2017	V	D	N	V	I	I	K	V	F	D	L	M
AD 435	Dog	Thailand	2017	V	D	N	V	I	I	K	V	F	D	L	M
AD 436	Dog	Thailand	2017	V	D	N	V	I	I	K	V	F	D	L	M
AD 518	Dog	Thailand	2017	V	D	N	V	I	I	K	V	F	D	L	M
AD 557	Dog	Thailand	2017	V	D	N	V	I	I	K	V	F	D	L	M
AD 564	Dog	Thailand	2017	V	D	N	V	I	I	K	V	F	D	L	M
AD 584	Dog	Thailand	2017	V	D	N	V	I	I	K	V	F	D	L	M
AD 20250	Dog	Thailand	2017	V	D	N	V	I	I	K	V	F	D	L	M
AD 20806	Dog	Thailand	2018	V	D	N	V	I	I	K	V	F	D	L	M
AD 21269	Dog	Thailand	2018	V	D	N	V	I	I	K	V	F	D	L	M
AD 21797	Dog	Thailand	2018	V	D	N	V	I	I	K	V	F	D	L	M
<b>Betacoronaviruses</b>															
BJ232	Dog	china	2014	V	D	N	V	I	I	K	V	F	D	L	M
K37	Dog	South Korea	2008	V	D	N	V	I	I	K	V	F	D	L	M
K39	Dog	South Korea	2008	V	D	N	V	I	I	K	V	F	D	L	M
02/005	Dog	Japan	2002	V	D	N	V	I	I	K	V	F	D	L	M
06/075	Dog	Japan	2006	V	D	N	V	I	I	K	V	F	D	L	M
K9	Dog	South Korea	2008	V	D	N	V	I	I	K	V	F	D	L	M
240/05	Dog	Italy	2005	V	D	N	V	I	I	K	V	F	D	L	M
4182	Dog	UK	2003	V	D	N	V	I	I	K	V	F	D	L	M
T101	Dog	UK	2003	V	D	N	V	I	I	K	V	F	D	L	M
BCoV—ENT	Bovine	USA	1998	T	N	H	I	V	T	Q	A	V	E	S	V
Oh-440-tc	Bovine	USA	1996	T	N	H	I	V	T	Q	A	V	E	S	V
DB2	Bovine	USA	1983	T	N	H	I	V	T	Q	A	V	E	S	V
AH187	Bovine	USA	2000	T	N	H	I	V	T	Q	A	V	E	S	V
Kakegawa	Bovine	Japan	1980	T	N	H	I	V	T	Q	A	V	E	S	V
<b>HCoV-OC43</b>															
Paris	Human	France	2001	T	N	H	I	V	T	Q	A	V	E	S	V
901-43	Human	USA	1990	T	N	H	I	V	T	Q	A	V	E	S	V
ATCC VR-759	Human	USA	1967	T	N	H	I	V	T	Q	A	V	E	S	V
<b>HECoV-4408</b>															
HECoV/4408	Human	Germany	1988	T	N	H	I	V	T	Q	A	V	E	S	V
HECoV/4408	Human	USA	2009	T	N	H	I	V	T	Q	A	V	E	S	V

\*Nucleotide positions were based on HCoV-OC43, BCoV and HeCoV

**Table 4.7.** Genetic analysis of S gene of Thai-CRCoVs and reference CRCoVs from the GenBank.

Virus	Host	Country	Year	S (4092 nt)				
				Asian CRCoV specific region			Esterase activity	Proteolytic cleavage
				D28N	A225V	V465A	37-40	KRRSRR
<b>CRCoV in this study</b>								
AD21	Dog	Thailand	2016	N	V	A	STDV	KRRSRR
AD 431	Dog	Thailand	2017	N	V	A	STDV	KRRSRR
AD 129	Dog	Thailand	2016	N	V	A	STDV	KRRSRR
AD 242	Dog	Thailand	2016	N	V	A	STDV	KRRSRR
AD 386	Dog	Thailand	2016	N	V	A	STDV	KRRSRR
AD 404	Dog	Thailand	2017	N	V	A	STDV	KRRSRR
AD 435	Dog	Thailand	2017	N	V	A	STDV	KRRSRR
AD 436	Dog	Thailand	2017	N	V	A	STDV	KRRSRR
AD 518	Dog	Thailand	2017	N	V	A	STDV	KRRSRR
AD 557	Dog	Thailand	2017	N	V	A	STDV	KRRSRR
AD 564	Dog	Thailand	2017	N	V	A	STDV	KRRSRR
AD 584	Dog	Thailand	2017	N	V	A	STDV	KRRSRR
AD 20250	Dog	Thailand	2017	N	V	A	STDV	KRRSRR
AD 20806	Dog	Thailand	2018	N	V	A	STDV	KRRSRR
AD 21269	Dog	Thailand	2018	N	V	A	STDV	KRRSRR
AD 21797	Dog	Thailand	2018	N	V	A	STDV	KRRSRR
<b>Reference CRCoV</b>								
BJ232	Dog	china	2014	N	V	A	STDV	KRRSRR
K37	Dog	South Korea	2008	N	V	A	STDV	KRRSRR
K39	Dog	South Korea	2008	N	V	A	STDV	KRRSRR
02/005	Dog	Japan	2002	D	A	V	STDV	KRRSRR
06/075	Dog	Japan	2006	D	A	V	STDV	KRRSRR
K9	Dog	South Korea	2008	N	V	A	STDV	KRRSRR
240/05	Dog	Italy	2005	D	A	V	STDV	KRRSRR
4182	Dog	UK	2003	D	A	V	STDV	QRRSRR
T101	Dog	UK	2003	D	A	V	STDV	QRRSRR

#### 4.5. Discussion

To our knowledge, this study is the first to report whole genome characterization of canine respiratory coronavirus (CRCoV) in dogs in Thailand. Up to date, there are only 3 complete whole genomes sequences of CRCoVs available in the GenBank database (strain; BJ232, K37 and CRCoV1). In this study whole genome sequences of 2 Thai-CRCoVs (AD-21, AD-431) as well as HE and S gene sequences of 14 Thai-CRCoVs will broaden the knowledge and genetic information of CRCoV in the database. Based on three year surveillance, the prevalence of CRCoVs in dogs with respiratory signs was 13.13% which was higher than the previous study in Thailand (2 % of CRCoV) (Piewbang et al., 2016). CRCoV is frequently be detected in dogs of young age (<1 year) and older dog (>5 years) which in agreement with other studies (Priestnall et al., 2006; Priestnall et al., 2007). CRCoVs could be detected year round but more frequently detected in winter season suggesting seasonal pattern of CRCoV infection in dogs (Erles and Brownlie, 2005; Maboni et al., 2019). CRCoV could be co-infected with other respiratory viruses such as CPIV-5 and CIV which is similar to previous studies (Lavan and Knesl, 2015; Maboni et al., 2019). It also has been reported that CRCoV co-infection with other viruses might associate with ciliary stasis and induce secondary infection (Erles et al., 2003b; Priestnall et al., 2014).

In general, the coronaviruses circulating in dogs are respiratory CoV (betacoronavirus group A) and enteric coronavirus CoV (alphacoronavirus) (Erles et al., 2003a) . Recently, the spillover of SARS-CoV2 (betacoronavirus group B) in dogs have also been reported (Patterson et al., 2020; Sit et al., 2020b). Phylogenetically, Thai CRCoVs (AD-21 and AD-431) were grouped into betacoronavirus group A which including BoV, HCoV-OC43 and HeCoV-4408 but in separated clusters from canine enteric coronavirus (CoV) of alphacoronavirus group and canine SARS-CoV-2 of betacoronavirus group B. It is noted that the three CRCoVs previously reported in China (BJ232), Sweden (CRCoV1) and South Korea (K37) were also clustered in betacoronavirus group A (Lim et al., 2013; Wille et al., 2020b). The genetic analysis showed that Thai-CRCoVs were closely related to CRCoV from South Korea and China with 98.70-99.80 % nucleotide identities (91.40 -93.60% amino acid identities). Based

on phylogenetic analysis of whole genome sequences, Thai-CRCoV were estimated to separate from HCoV-OC43 and BCoV with the most recent common ancestor since 2004, suggesting that Thai-CRCoVs could have share common origin with BCoV and HCoV-OC-43 (Vijgen et al., 2005; Wille et al., 2020b). It could be speculated that Thai-CRCoVs potential originated from interspecies transmission between dog, human and bovine. This observation is in agreement with the previous study that CRCoV (K37) shares common origin with BCoV (JBJ)(Lu et al., 2017). In this study, the nucleotide substitution rate of S gene of the Thai CRCoVs was  $7.8818 \times 10^{-5}$  substitution per site per year (95% posterior densities (HPD)  $2.3042 \times 10^{-5}$ - $1.3619 \times 10^{-4}$  which higher than most RNA virus ( $1 \times 10^{-5}$  substitution rate) (Firth et al., 2010). It has been documented that coronavirus have high mutation rate that can result in novel or virulent viruses and subsequent cross-species transmission for example MERS, SARS-CoV and SARS-CoV-2 (Fung et al., 2020; Ye et al., 2020). Since Thai-CRCoVs were grouped in betacoronavirus group A which closely related to HCoV-OC43 and BCoV. It has been reported that the CRCoV, HCoV and BCoV can bind to the same sialic acid receptor for host cell entry suggesting that HCoV-OC43 could have been originated from animal (Szczepanski et al., 2019; Vijgen et al., 2005). Thus, the monitoring of genetic diversity and possibly cross-species transmission of CRCoVs should not be ignored.



#### 4.6. Conclusion

In conclusion, this study demonstrated the first reported genetic of whole genome sequence and genetic characteristic of canine respiratory coronavirus (CRCoV) recovered from dogs in Thailand. Thai-CRCoVs could be grouped into betacoronavirus group A, but in separated groups from canine enteric coronavirus (CoV) of alphacoronavirus group and canine SARS-CoV-2 of betacoronavirus group B. The genetic and MCC tree analysis showed that Thai-CRCoVs were closely related to CRCoV from South Korea and China as well as human coronavirus (HCoV-OC43) and bovine coronavirus (BCoV). It could be speculated that Thai-CRCoVs possibly originated from interspecies transmission between dog, human and bovine. Thus, persons at risk especially those who in close contact with domestic dogs should pay more attention to possible zoonotic infection of the viruses. The survey and characterization of CRCoV should be investigated on a larger scale to determine the distribution, genetic diversity, and possibly cross-species transmission of CRCoVs in the future.

## CHAPTER V

### CANINE KOBUVIRUS

Parts of this work have been published in

#### First Detection and Genetic Characterization of Canine Kobuvirus in domestic dogs in Thailand

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#### 5.1 Abstract

Canine Kobuvirus (CaKoV) has been detected both in healthy and diarrheic dogs and in asymptomatic wild carnivores. In this study, we conducted a survey of CaKoV at small animal hospitals in Bangkok and vicinity of Thailand during September 2016 to September 2018. Three hundred and seven rectal swab samples were collected from healthy dogs (n=55) and dogs with gastroenteritis symptoms (n=252). Of 307 swab samples tested by using one-step RT-PCR specific to 3D gene, we found CaKoV positivity at 17.59% (54/307). CaKoVs could be detected in both sick (19.44%) and healthy (9.09%) animals. In relation to age group, CaKoV could be frequently detected in younger dogs (25.45%). Our result showed no seasonal pattern of CaKoV infection in domestic dogs. In this study, we characterized CaKoVs by whole genome sequencing (n=4) or 3D and VP1 gene sequencing (n=8). Genetic and phylogenetic analyses showed that whole genomes of Thai CaKoVs were closely related to Chinese CaKoVs with highest 99.5% amino acid identity suggesting possible origin of CaKoVs in Thailand. In conclusion, this study was the first to report the detection and genetic characteristics of CaKoVs in domestic dogs in Thailand. CaKoVs could be detected in both sick and healthy dogs. The virus is frequently detected in younger dogs. Thai CaKoVs were genetically closely related and grouped with

Chinese CaKoVs. Our result raises the concerns to vet practitioners that diarrhea in dogs due to Canine Kobuvirus infection should not be ignored.



## 5.2 Introduction

Kobuvirus (KoV) is a single-strand positive-sense RNA virus. KoV belongs to the family *Piconaviridae*, genus *Kobuvirus*, which consists of four species Aichivirus A, B, C and D (Adams et al., 2016; Oem et al., 2014b; Yamashita et al., 2003). KoV has been reported in feces of several mammal species including humans, ruminants, pigs, dogs, cat, bat and rodents (Carmona-Vicente et al., 2013; Khamrin et al., 2009; Li et al., 2010a; Lu et al., 2018; Mohamed et al., 2018; Phan et al., 2011; Yamashita et al., 2003). The Kobuvirus species Aichivirus A contains four types including Aichi virus 1, Canine Kobuvirus 1 (CaKoV), Feline Kobuvirus 1 (FeKoV) and Murine Kobuvirus 1 (MuKoV). Canine Kobuvirus 1 (CaKoV) was first reported in dogs with acute gastroenteritis in the US in 2011 (Kapoor et al., 2011; Li et al., 2011). CaKoV was subsequently reported in dogs in UK, Italy, Australia, Japan, Korea, and China (Carmona-Vicente et al., 2013; Di Martino et al., 2013; Kong et al., 2016; Oem et al., 2014a; Soma et al., 2016b). The virus was reported in wild carnivore (Jackal and Hyena) and domestic dogs in Tanzania, Africa (Olarte-Castillo et al., 2015), in foxes in Spain (Bodewes et al., 2014) and in foxes (Di Martino et al., 2014) and wolves in Italy (Melegari et al., 2018). Several studies have reported the detection of CaKoV infection in dogs with or without diarrhea and sometime systemic infection (Ribeiro et al., 2017). To date, only 12 completed CaKoV genomes are available in the GenBank database.

During September 2016 to September 2018, the center of excellence for emerging and re-emerging infectious diseases in animals (CUEIDAs), Chulalongkorn University conducted a survey of Canine Kobuvirus in domestic dogs at small animal hospitals in 5 provinces of Thailand. The survey was conducted under the Chulalongkorn University's animal use and care protocol # 1731074. The result of this study provided the first detection and genetic characterization of CaKoV isolated from domestic dogs in Thailand.

## 5.3 Material and Methods

### 5.3.1. Sample collection

Sample collection was conducted in domestic dogs at small animal hospitals in Bangkok and vicinity of Thailand During September 2016 to September 2018. 307 rectal swab samples were collected from healthy dogs (n=55) and dogs with gastroenteritis symptoms (n=252) including vomiting, watery diarrhea, hemorrhagic diarrhea and dehydration. The swab samples were collected from dogs of young age (< 1 year) (n=165), adult (1-5 years) (n=98) and older (>5 years) (n=44). The animal demographic data including age, sex, breed, and vaccination history were also recorded. The ethics was conducted under the Chulalongkorn University's animal use and care protocol # 1731074. The consent to participate of the owners of the animals used in this study was obtained in writing.

### 5.3.2. Canine Kobuvirus (CaKoV) detection

All 307 samples were subjected to Canine Kobuvirus identification by one step RT-PCR using primers specific to 3D gene of CaKoV (Choi et al., 2015). First, RNA extraction was performed using the QIAasympyony DSP viral/Pathogen mini kit (Qiagen, Hilden, Germany) following manufacturer's instructions. To detect CaKoV, RNA samples were screened for 3D gene of CaKoV by using one step RT-PCR assay. The primers used in this study were previously described including U 1 F (5' -CATGCTCCTCGGTGGTCTCA-3') and U1R (5'-GTCCGGGTCCATCACAGGGT -3') (Choi et al., 2015). Briefly, one-step RT-PCR was conducted in a total final volume of 25 µl comprising 3 µl of template RNA, 15 µl of 2xReaction Mix (Invitrogen, USA), 0.6 µl of 10 µM forward and reverse primers, 1.2 µl of SuperScript III RT (Invitrogen, USA) and distilled water to final volume 25 µl. The condition of RT-PCR assay included cDNA synthesis step at 55°C for 30 minutes, next to an initial denaturation step at 94 °C for 2 min, following 40 cycles of denaturation at 94 °C for 30 seconds, annealing at 52 °C for 30 seconds and extension at 68 °C for 1 minute, as well as, final extension step at 68 °C for 5 minutes. To confirm CaKoV, 4 µl of PCR products were run on a 1.5% agarose gel, which mixed with Red Safe at 100 volts for 45 min. The expected size of

CaKoV positive amplified products was 631 bp. Due to dogs showed clinical signs similar to other canine viral enteric diseases, all samples were also tested for Canine Parvovirus (n=307), Canine Rotavirus (n=307) and Canine Coronavirus (n=30) (Buonavoglia et al., 2001b; Ortega et al., 2017a; Pratelli et al., 1999).

### 5.3.3. Canine Kobuvirus characterization

In this study, four CaKoV positive samples (CU-53, CU-101, CU-247 and CU-716) were selected for whole genome sequencing and additional eight CaKoV positive samples were selected for 3D and VP1 gene sequencing. The CaKoVs were selected based on epidemiological and demographic data such as age, date of isolation, breed, and vaccination history. For sequencing, nucleotide sequences of each gene of the viruses were amplified by new primer sets designed by using Primer 3 plus program (Koressaar and Remm, 2007a). List of oligonucleotide primers is provided in Table 5.1. In brief, PCR was proceed in a final volume of 30  $\mu$ l containing 2  $\mu$ l of cDNA, 0.4  $\mu$ M of each forward and reverse primer, 1X TopTaq Master Mix, 1X Coral Load, and distilled water. The PCR condition was set as initial denaturation at 94°C for 3 minutes; 40 cycles of denaturation at 94°C for 30 seconds, annealing at 50-55°C for 45 seconds, extension at 72°C for 1-1.30 minutes; and final extension at 72°C for 7 minutes. PCR products were then purified and sequenced (1<sup>st</sup> Base Laboratories Sdn Bhd, Malaysia). Nucleotide sequences were edited, validated and assembled by using SeqMan software v.5.03 (DNASTAR Inc.; Wisconsin, USA).

**Table 5.1.** Oligonucleotide primers used for CaKoV whole genome sequencing.

Primer	Nucleotide sequence (5'-3')	Nucleotide position
CaKoV-1F	TGTGCCCAATCTCTTGACTCC	10
CaKoV-1R	GTGGCAATAAGGACACGGGA	769
CaKoV-2F	CCCGTGCCTTATTGCCACT	886
CaKoV-2R	GCCTTCCGGCGAGTTTCCC	1558
CaKoV-3F	ACAGCTCCTCAAATTCCTCCG	1181
CaKoV-3R	ACAAAGGGGGAGTTCTTGGC	2527
CaKoV-4F	CACTGGAAAACCCGGATCG	2399
CaKoV-4R	GTCAGGGACAGGGATGGAG	3273
CaKoV-5F	CCTCATCCAAGGCTTCTTTT	2974
CaKoV-5R	ACTGTACTCCCACGGTTTGC	4054
CaKoV-6F	CCAATCCGGAAAAATCTGTG	3803
CaKoV-6R	ATGTGCATCAGCAAGTTTGG	4847
CaKoV-7F	ATCTCTGGACTCCTCGTCATC	4575
CaKoV-7R	GATGAGCTCGTCCAGGTTG	5655
CaKoV-8F	ACACCTCCCGAGTGATTGTC	5394
CaKoV-8R	CGTAGAGGGGGGCAGCCTTG	6394
CaKoV-9F	CCTCCAAGGCTGTCATGTCT	6171
CaKoV-9R	GTCCAGTGCACGTCGGGG	7296
CaKoV-10F	GATCCGGATTATGTCTACTCCAC	7105
CaKoV-10R	CAGTTAGAAAAGTTCAAAGACAACC	8287

#### 5.3.4. Phylogenetic and genetic analyses of Canine Kobuviruses

The phylogenetic and genetic analyses were performed by comparing nucleotide sequences of Thai CaKoVs with those of Kobuvirus available from the GenBank database. The reference nucleotide sequences of CaKoVs were retrieved based on their different geographic locations, host species and date of isolation. Phylogenetic analysis of CaKoV was performed by using MEGA v.6.0 (Tempe, AZ, USA) (Tamura et al., 2013) with neighbor-joining method with Kimura 2-parameter with 1,000 bootstrap replicates and Beast program with Bayesian Markov chain Monte Carlo (BMCMC) with 10,000,000 generations and an average standard deviation of split frequencies  $<0.05$  (Drummond et al., 2012b). For genetic analysis, the nucleotide sequences and deduced amino acids of CaKoV were aligned and compared using MegAlign software v.5.03 (DNASTAR Inc.; Wisconsin, USA). Pairwise comparison of nucleotides and amino acids of Thai CaKoV and those of reference CaKoVs were conducted. The variable and unique amino acids related to receptor binding of the viruses and host preferences of CaKoVs were monitored.



## 5.4 Results

### 5.4.1. Canine Kobuviruses in domestic dogs in Thailand

During September 2016 to September 2018, we conducted a survey of viral enteric diseases in domestic dogs in small animal hospitals in 5 provinces of Thailand (Bangkok, Nakhon Ratchasima, Ratchaburi, Suphanburi, and Tak). We tested 307 rectal swab samples for CaKoV by using one-step RT-PCR specific to 3D gene. Based on a two-year survey, we found CaKoV positivity at 17.59% (54/307). CaKoVs could be detected in both sick (19.44% (49/252)) and healthy (9.09% (5/55)) animals. Our result showed no seasonal pattern of CaKoV infection in dogs (Figure 5.1 and 5.2). In relation to age group, CaKoV could be frequently detected in younger dogs at 25.45% (42/165) (Table 5.2). The coinfections of CaKoV with other enteric viral pathogens were observed including CaKoV/Canine parvovirus/Canine Coronavirus (n = 6), CaKoV/Canine parvovirus (n = 20) and CaKoV/Canine Coronavirus (n = 2). In this study, 12 CaKoVs were selected and characterized by whole genome sequencing (n=4) or 3D and VP1 gene sequencing (n=8). The viruses were selected to represent epidemiological and demographic data such as age, date of isolation and breed. In this study, nucleotide sequences of the CaKoV were submitted to the GenBank database under the accession numbers MK201776 - MK201795 (Table 5.3).

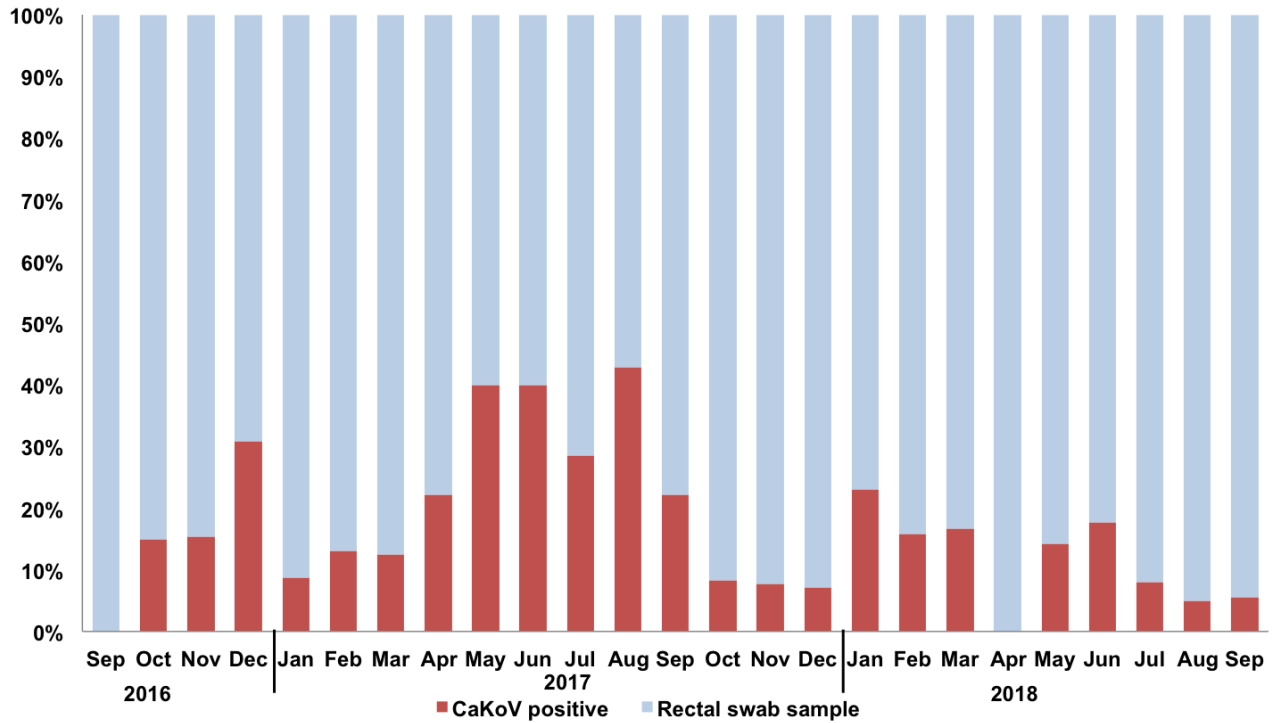
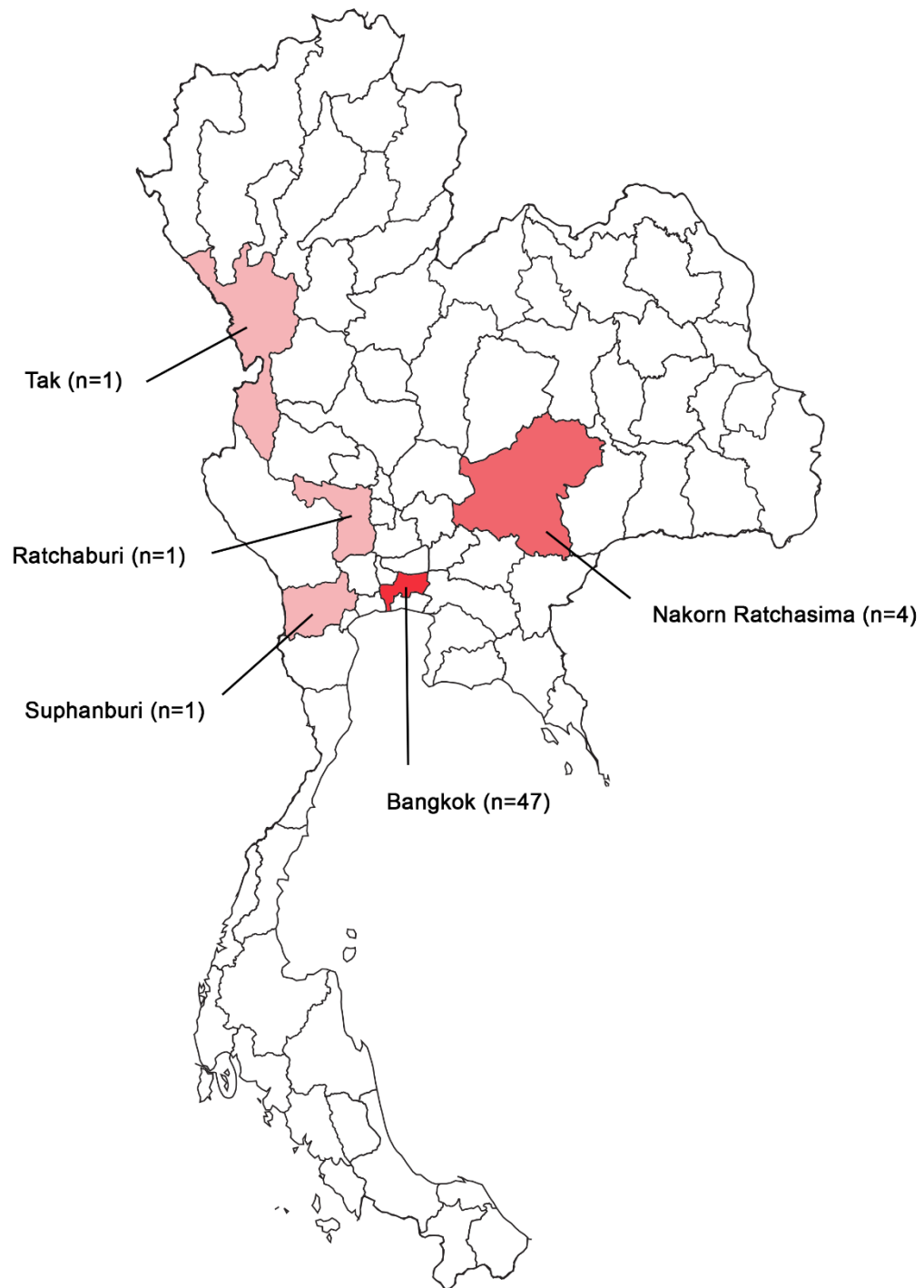


Figure 5.1. Number of samples collected and CaKoVs detected by month in this study.





**Figure 5.2.** Number of samples collected and CaKoVs detected by provinces in this study (Map of Thailand with permission by World Trade Press)

**Table 5.2.** Association of age of CaKoVs detection in this study

Age	CaKoVs positive (%)		
	Asymptomatic	Clinical sign	Total
Young (< 1 year)	2/13 (15.38%)	40/152 (26.32%)	42/165 (25.45%)
Adult (1-5 years)	3/38 (7.89%)	1/60 (1.67%)	4/98 (4.08)
Older (>5 years)	0/4 (0%)	8/40 (20.00%)	8/44 (18.18)
	5/55 (9.09%)	49/252 (19.44%)	



Table 5.3. Detail description of Thai CaKoVs characterized in this study.

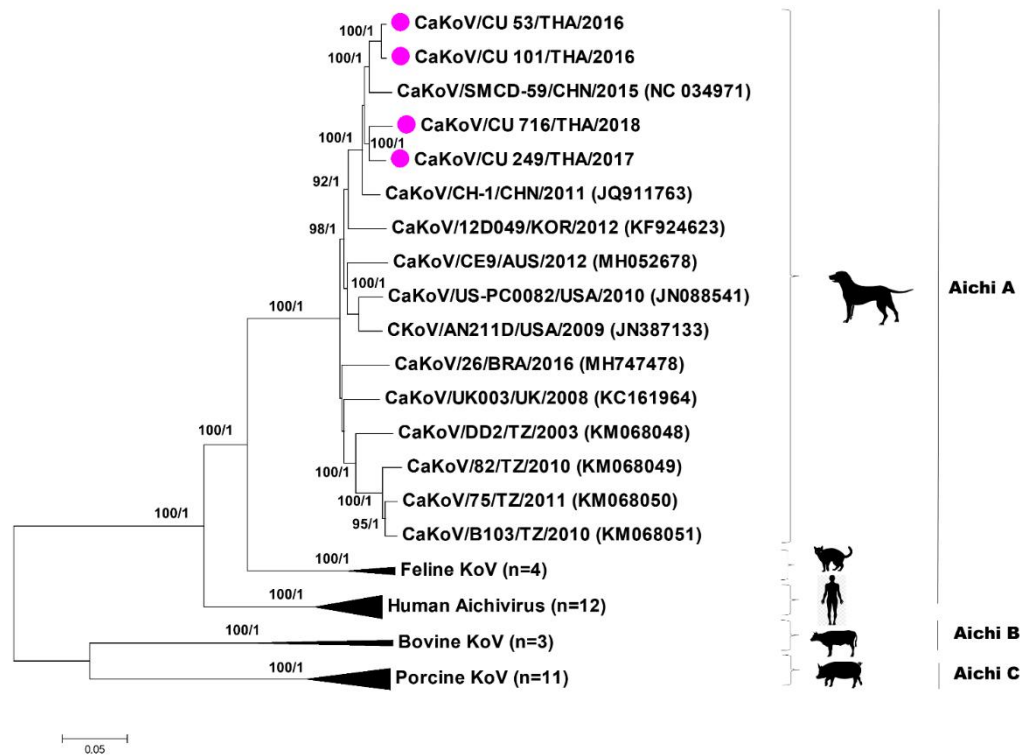
Virus	Date	Location	Region	Age	Breed	Clinical signs	Sequencing	GenBank
							ng	Accession #
CU-53	Oct-16	Bangkok	Central	2 month	Pomeranian	Diarrhea	WG*	MK201776
CU-101	Dec-16	Bangkok	Central	3 month	Pekingese	Diarrhea	WG	MK201777
CU-249	May-17	Bangkok	Central	3 month	Pomeranian	Diarrhea	WG	MK201778
CU-716	Jan-18	Bangkok	Central	12 years	Shizu	Diarrhea	WG	MK201779
CU-83	Nov-16	Bangkok	Central	2 month	Pomeranian	Diarrhea	3D, VP1**	MK201780, MK201788
CU-100	Dec-16	Ratchaburi	Central	6 month	Great Dane	Diarrhea	3D, VP1	MK201781, MK201789
CU-125	Jan-17	Tak	Northern	2 month	Bang Keaw	Asymptomatic	3D, VP1	MK201782, MK201790
CU-224	Feb-17	Bangkok	Central	9 years	Pomeranian	Diarrhea	3D, VP1	MK201783, MK201791
CU-241	Apr-17	Bangkok	Central	3 month	Mixed	Diarrhea	3D, VP1	MK201784, MK201792
CU-250	May-17	Bangkok	Central	3 month	Pomeranian	Diarrhea	3D, VP1	MK201785, MK201793
CU-260	Jun-17	Nakhon Ratchasima	North-Eastern	2 month	German Shepherd	Diarrhea	3D, VP1	MK201786, MK201794
CU-273	Aug-17	Bangkok	Central	2 month	Pomeranian	Diarrhea	3D, VP1	MK201787, MK201795

\* WG: Whole genome sequencing

\*\* 3D, VP1: 3D and VP1 gene sequencing

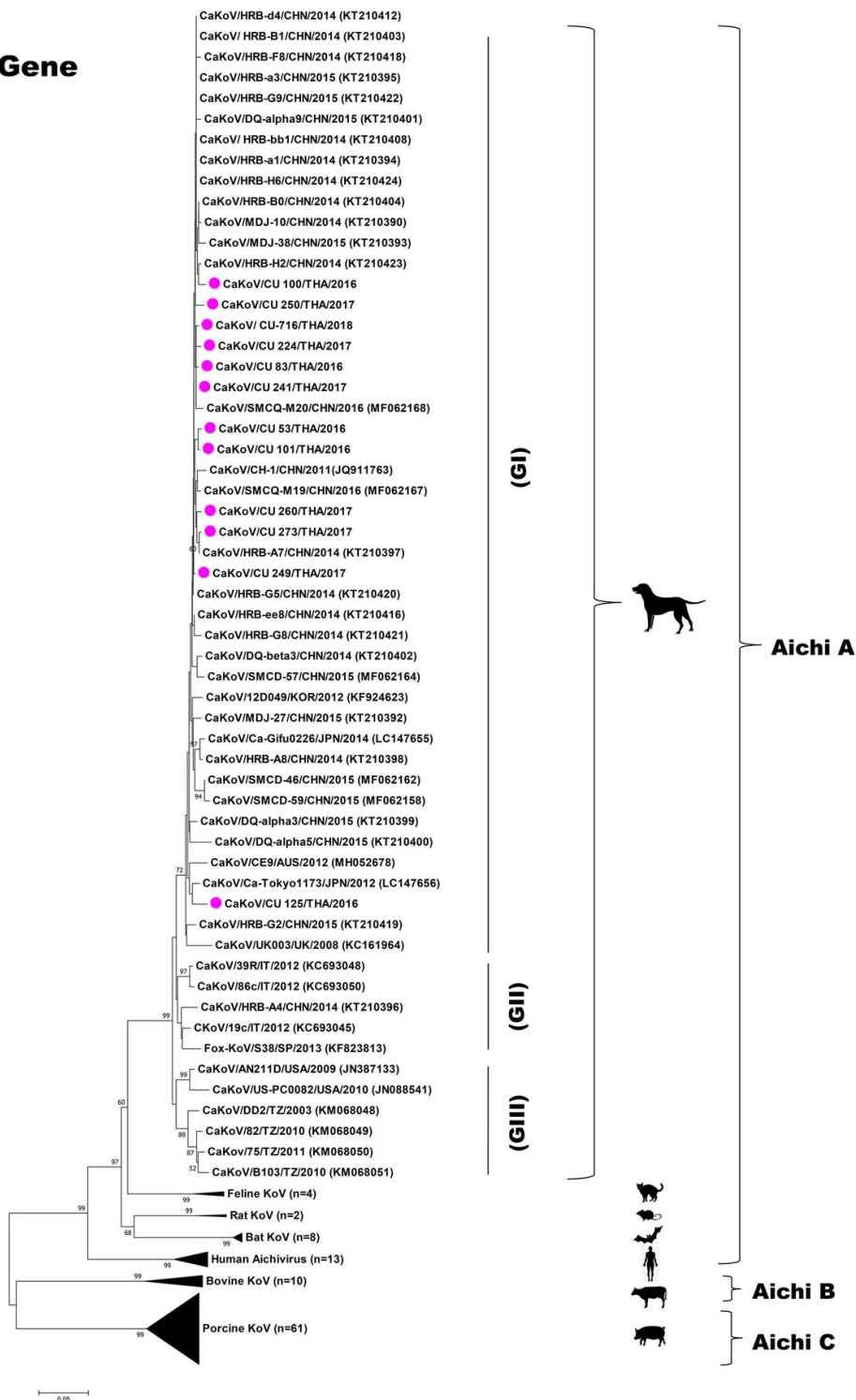
#### 5.4.2. Phylogeny of the Thai Canine Kobuviruses

Phylogenetic analysis of whole genome of CaKoVs showed that the Thai CaKoVs were closely related to each other and clustered with Aichivirus A. The cluster Aichivirus A contains Kobuviruses from dogs, cats, rodents, bats and human. While Aichivirus B and C contain Kobuviruses from cattle and pigs, respectively. Based on whole genome sequence, Thai CaKoVs were closely related to Chinese CaKoVs sub-cluster but in separated sub-cluster from the viruses from the US, UK, Brazil and Tanzania (Figure 5.3). Phylogenetic analysis of 3D and VP1 of Thai CaKoVs and reference CaKoVs from various animal species were also performed. Similarly, 3D gene of Thai CaKoVs were grouped together with Chinese CaKoVs (G1 sub-cluster) but separated from the viruses in sub-clusters of sub-clusters G2 as well as G3 (Figure 5.4). Phylogenetic analysis of VP1 gene, the viruses can be clustered into 2 major subgroups, US/EU/Africa subgroup and China/Thailand subgroup (Figure 5.5).



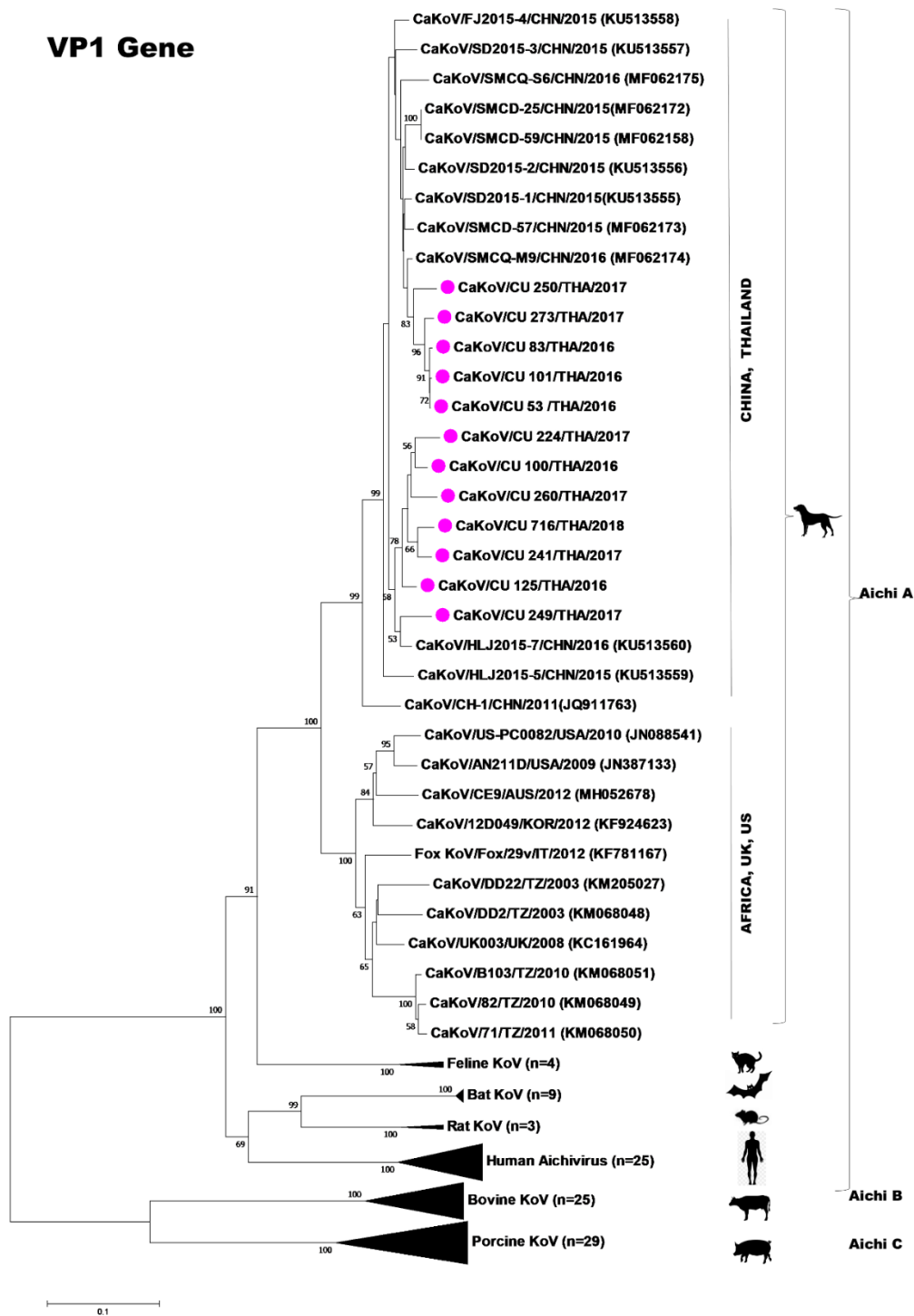
**Figure 5.3.** Phylogenetic tree of the completed genome of CaKoVs. The phylogenetic tree was constructed by using MEGA v6.0 with neighbor-joining algorithm with Kimura-2 parameter model and Beast program with Bayesian Markov chain Monte Carlo (BMCMC) with 10,000,000 generations and an average standard deviation of split frequencies <0.05. Values on branches represent bootstrap and posterior probability values.

**3D Gene**



**Figure 5.4.** Phylogenetic tree of 3D gene of CaKoVs. The phylogenetic tree was constructed by using MEGA v 6.0 with neighbor-joining algorithm with Kimura-2 parameter model with 1,000 replications of bootstrap analysis.





**Figure 5.5.** Phylogenetic tree of VP1 gene of CaKoVs. The phylogenetic tree was constructed by using MEGA v6.0 with neighbor-joining algorithm with Kimura-2 parameter model with 1,000 replications of bootstrap analysis.

### 5.4.3. Genetic analysis of the Thai Canine Kobuviruses

We compared the nucleotide and deduced amino acid sequences of Thai CaKoVs against those of reference viruses from the US, UK, Italy, China, and Korea (Table 5.4 and 5.5). Our results showed that whole genome of 4 Thai CaKoVs (CU-53, CU-101, CU-249 and CU-716) shared 96.7-99.3% nucleotide similarity (99.6-100% amino acid similarity) to each other and posed highest nucleotide similarity to Chinese CaKoVs including SMCD-59 (97.0% nt and 99.5% aa identity) and CH-1 (96.8% nt and 98.7% aa identity). Our analysis showed that the VP1 protein was the most diverse gene with 93.4 -99.9 % nucleotide similarity (96.9-100% aa similarity) among Thai CaKoVs and 82.2-96.8% with other reference CaKoVs. The most variable region of VP1 is position 201-243, especially proline rich region. Putative proline rich region at VP1-228-240 (P<sub>228</sub>XPPPPXPPXP<sub>240</sub>) was also observed in Thai CaKoVs as well as reference viruses (Table 5.6). In this study, unique amino acids were found in Thai and Chinese CaKoVs at the position, 65V, 67D, 199L, 138T, 150P, 151M, 153D, 201S, 204Q, 205Q, 210Q, 213T and 241E (Table 5.4). Analysis of predicted amino acid cleavage sites of whole genome were conserved among Thai CaKoVs (Table 5.7).

**Table 5.4.** Pairwise comparison of whole genome of Thai CaKovs (CU-101) and reference CaKovs

Virus	Accession number	Year	Country	% nucleotide identity (% amino acid identity)													
				WGS	VP0	VP3	VP1	2A	2B	2C	3A	3B	3C	3D			
CaKov/CU-101/THA/2016	This study*	2016	Thailand	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)
CaKov/CU-53/THA/2016	This study*	2016	Thailand	99.3 (100)	99.8 (100)	99.3 (100)	99.5 (100)	100 (100)	97.1 (100)	99.4 (100)	99.4 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	98.8 (100)
CaKov/CU-249/THA/2017	This study*	2017	Thailand	96.7 (99.6)	96.2 (100)	97.2 (99.6)	94.3 (98.2)	100 (100)	97 (100)	97.2 (100)	97.2 (100)	97.2 (100)	97.2 (98.9)	96.3 (100)	97.6 (99.7)	97.5 (100)	97.5 (100)
CaKov/CU-716/THA/2018	This study*	2018	Thailand	96.7 (99.8)	95.2 (100)	97.5 (99.6)	95.2 (99.6)	100 (100)	96 (100)	96.8 (100)	96.8 (100)	96.8 (98.9)	96.8 (98.9)	96.3 (96.3)	98.3 (99.7)	97.5 (100)	97.5 (100)
CaKov/CH-1/CHN/2011	JO911763	2011	China	96.8 (98.7)	97.4 (99.7)	97.6 (99.6)	91.2 (91.8)	98.3 (100)	97.1 (100)	97.3 (99.7)	97.3 (99.7)	98.6 (100)	98.6 (100)	100 (100)	98.5 (99.7)	97.1 (100)	97.1 (100)
CaKov/SMCD-59/CHN/2015	NC034971	2015	China	97 (99.5)	92.5 (98.7)	93 (99.6)	96.5 (97.8)	100 (100)	95.3 (100)	96 (100)	96 (100)	94.7 (97.9)	94.7 (97.9)	89 (96.3)	95.6 (99.2)	95.3 (99.6)	95.3 (99.6)
CaKov/12D049/KOR/2012	KF924623	2012	Korea	94.2 (97.9)	93.2 (98.7)	93.2 (99.6)	85.5 (90)	100 (100)	97.3 (100)	96.4 (99.1)	96.4 (99.1)	97.2 (98.9)	97.2 (98.9)	93.9 (96.3)	96.9 (98.7)	94.7 (98.5)	94.7 (98.5)
CaKov/UK003/UK/2008	KC161964	2008	UK	93.6 (98.1)	92.4 (99)	95.2 (99.6)	86 (89.6)	100 (100)	93.4 (99)	95.3 (99.7)	95.3 (99.7)	94.3 (98.9)	94.3 (98.9)	91.5 (96.3)	95.9 (99.2)	96.4 (100)	96.4 (100)
CaKov/26/BRA/2016	MH747478	2016	Brazil	92.8 (97.9)	91.1 (99)	91.7 (99.1)	83.2 (86)	100 (100)	95.6 (100)	95.8 (100)	95.8 (100)	96.5 (98.9)	96.5 (98.9)	87.8 (96.3)	94.7 (98.2)	96.5 (99.6)	96.5 (99.6)
CaKov/US-PC0082/USA/2010	JN088541	2010	USA	93.4 (97.7)	91.1 (97.4)	92.3 (99.1)	85.7 (88.2)	100 (100)	93.3 (98)	94.2 (99.4)	94.2 (99.4)	92.9 (98.9)	92.9 (98.9)	89 (96.3)	94.6 (99)	94.4 (98.9)	94.4 (98.9)
CaKov/CE9/A/US/2012	MH052678	2012	Australia	93.7 (97.6)	97.6 (99.5)	96.4 (100)	85.7 (90)	100 (100)	97.6 (100)	97.6 (100)	97.6 (100)	96.8 (98.9)	96.8 (98.9)	95.1 (100)	97.4 (99.5)	97 (100)	97 (100)
CaKov/75/TZ/2011	KM068050	2011	African	92.1 (97.5)	92.5 (99.2)	92.4 (99.6)	84.2 (88.5)	100 (100)	96.3 (99.5)	95.6 (99.4)	95.6 (99.4)	95 (98.9)	95 (98.9)	90.2 (96.3)	96.4 (99.2)	96.5 (99.6)	96.5 (99.6)
CaKov/B103/T/Z/2010	KM068051	2010	African	92.2 (97.5)	90.8 (96.9)	91.8 (98.7)	84.7 (89.2)	100 (100)	93.4 (99)	93.8 (99.4)	93.8 (99.4)	92.9 (98.9)	92.9 (98.9)	89 (96.3)	94.6 (99.2)	94.9 (99.3)	94.9 (99.3)
CaKov/DD2/T/Z/2003	KM068048	2003	African	92.3 (97.9)	91 (98.7)	94.1 (99.6)	84.3 (89.2)	100 (100)	93.4 (99)	93.2 (98.8)	93.2 (98.8)	92.6 (98.9)	92.6 (98.9)	89 (96.3)	94.9 (99.2)	94.9 (99.6)	94.9 (99.6)
CaKov/82/TZ/2010	KM068049	2010	African	91.8 (96.5)	91 (97.1)	92.4 (99.1)	84.2 (87.8)	100 (100)	93.3 (98)	92.8 (98.8)	92.8 (98.8)	92.2 (97.9)	92.2 (97.9)	91.5 (96.3)	94.4 (98.7)	94.4 (98.9)	94.4 (98.9)

**Table 5.5.** Pairwise comparison of 3D and VP1 genes of Thai CaKoVs (CU-101) and reference CaKoVs

Viruses	Accession number	Year	Country	% nucleotide identity (% amino acid identity)	
				3D	VP1
CaKoV/CU-101/THA/2016	This study*	2016	Thailand	100 (100)	100 (100)
CaKoV/CU-53/THA/2016	This study*	2016	Thailand	99.5 (100)	99.9 (100)
CaKoV/CU-83/THA/2016	This study*	2016	Thailand	98.8 (100)	99.7 (100)
CaKoV/CU-100/THA/2016	This study*	2016	Thailand	97.9 (100)	93.6 (97.8)
CaKoV/CU-125/THA/2016	This study*	2016	Thailand	97.1 (98.6)	94.9 (97.8)
CaKoV/CU-224/THA/2017	This study*	2017	Thailand	98.6 (100)	93.6 (97.8)
CaKoV/CU-241/THA/2017	This study*	2017	Thailand	99.0 (100)	94.5 (98.7)
CaKoV/CU-249/THA/2017	This study*	2017	Thailand	98.8 (100)	93.6 (97.4)
CaKoV/CU-250/THA/2017	This study*	2017	Thailand	98.1 (100)	96.6 (96.9)
CaKoV/CU-260/THA/2017	This study*	2017	Thailand	98.6 (100)	93.4 (96.9)
CaKoV/CU-273/THA/2017	This study*	2017	Thailand	98.6 (100)	98.5 (99.1)
CaKoV/CU-716/THA/2018	This study*	2018	Thailand	98.8 (100)	94.3 (98.7)
CaKoV/26/BRA/2016	MH747478	2016	Brazil	97.1 (99.3)	82.2 (84.2)
CaKoV/CE9/AUS/2012	MH052678	2012	Australia	97.6 (100)	83.7 (87.7)
CaKoV/B103/TZ/2010	KM068051	2010	African	93.6 (98.6)	84.8 (88.2)
CaKov/75/TZ/2011	KM068050	2011	African	94.0 (97.9)	83.8 (86.4)
CaKoV/82/TZ/2010	KM068049	2010	African	94.5 (98.6)	84.3 (86.4)
CaKoV/DD2/TZ/2003	KM068048	2003	African	94.8 (99.3)	84.0 (87.7)
CaKoV/UK003/UK/2008	KC161964	2008	UK	96.0 (100)	85.3 (88.2)
CaKoV/US-PC0082/USA/2010	JN088541	2010	USA	94.0 (99.3)	84.5 (86.4)
CaKoV/AN211D/USA/2009	JN387133	2009	USA	95.2 (99.3)	84.4 (86.8)
CaKoV/86c/IT/2012	KC693050	2012	Italy	96.0 (99.3)	N/A
CKoV/19c/IT/2012	KC693045	2012	Italy	96.2 (99.3)	N/A
CaKoV/Ca-Gifu0226/JPN/2014	LC147655	2014	Japan	97.6 (99.3)	N/A
CaKoV/Ca-Tokyo1173/JPN/2012	LC147656	2012	Japan	97.9 (100)	N/A
CaKoV/12D049/KOR/2012	KF924623	2012	Korea	97.1 (100)	84.7 (89.0)
CaKoV/CH-1/CHN/2011	JQ911763	2016	China	97.9 (100)	91.3 (89.9)
CaKoV/SMCD-59/CHN/2015	MF062158	2015	China	97.1 (100)	96.4 (96.9)
CaKoV/SMCD-57/CHN/2015	MF062173	2015	China	97.9 (100)	96.8 (97.8)

**Table 5.6.** Genetic analysis of Thai CaKovs compared with reference CaKovs at proline rich region

Viruses	Accession number	Amino acid at position													Proline rich region (228-240)
		65	67	119	138	150	151	153	201	204	205	210	213	241	
CaKov/CU-101/THA/2016	This study*	V	D	L	T	P	M	D	S	Q	Q	Q	T	E	PRAPPPPLPPLPTP
CaKov/CU-53/THA/2016	This study*	V	D	L	T	P	M	D	S	Q	Q	Q	T	E	PRAPPPPLPPLPTP
CaKov/CU-249/THA/2017	This study*	V	D	L	T	P	M	D	S	Q	Q	Q	T	E	PRAPPPPLPPLPTP
CaKov/CU-20176/THA/2018	This study*	V	D	L	T	P	M	D	S	Q	Q	Q	T	E	PRAPPPPLPPLPTP
<b>Reference</b>															
CaKov/SMCQ-M9/CHN/2016	MF062174	V	D	L	T	P	M	D	S	Q	Q	Q	T	E	PRAPPPPLPPLPTP
CaKov/SMCD-59/CHN/2015	NC 034971	V	D	L	T	P	M	D	S	Q	Q	Q	T	E	PRAPPPPLPPLPTP
CaKov/12D049/KOR/2012	KF924623	L	N	V	M	S	E	N	T	V	E	S	S	A	PRAPPPPLPPLPTP
CaKov/CE9/AUS/2012	MH052678	L	N	V	M	S	E	N	T	V	E	S	S	T	PRAPP_LPPLPTP
CaKov/AN211D/USA/2009	JN387133	L	N	P	M	S	E	N	T	V	E	S	S	A	PRAPPPPLPPLPTP
CaKov/US-PC0082/USA/2010	JN088541	L	N	V	M	S	E	N	T	V	E	S	S	A	<u>CPV</u> PPPLPPLPTP
CaKov/UK003/UK/2008	KC161964	L	N	V	M	S	E	N	T	V	E	S	S	T	PRAPPPPLPPLPTP
CaKov/26/BRA/2016	MH747478	L	N	V	M	S	E	N	T	V	E	S	S	T	<u>HGAP</u> PPPLPPLPTP
CaKov/75/TZ/2011	KM068050	L	N	V	M	S	E	N	T	A	E	S	S	T	<u>CPV</u> PPPLPPLPTP
CaKov/82/TZ/2010	KM068049	L	N	V	M	S	E	N	T	A	E	S	S	T	<u>CPV</u> PPPLPPLPTP
CaKov/B103/TZ/2010	KM068051	L	N	V	M	S	E	N	T	A	E	S	S	T	PRAPPPPLPPLPTP
CaKov/DD2/TZ/2003	KM068048	L	N	V	M	S	E	N	T	V	E	S	S	T	PRAPPPPLPPLPTP

**Table 5.7.** Genetic analysis of Thai CaKoVs compared with reference CaKoVs at putative amino acid cleavage sites.

Viruses	Year	Country	Amino acid position													
			171/172	553/554	776/777	1054/1055	1165/1166	1330/1331	1665/1666	1759/1760	1786/1787	2176/2177				
CU-53	2016	Thailand	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G	Q/G	Q/G	
CU-101	2016	Thailand	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G	Q/G	Q/G	
CU-249	2017	Thailand	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G	Q/G	Q/G	
CU-716	2018	Thailand	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G	Q/G	Q/G	
12D049	2012	Korea	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G	Q/G	Q/G	
UK003	2008	UK	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G	Q/G	Q/G	
26/BRA	2016	Brazil	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G	Q/G	Q/G	
SMCD-59	2015	China	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G	Q/G	Q/G	
CE9	2012	Australia	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G	Q/G	Q/G	
B103	2010	Africa	Q/G	Q/H	<u>Q/L*</u>	Y/V	Q/G	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G	Q/G	Q/G	
75	2011	Africa	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G	Q/G	Q/G	
82	2010	Africa	Q/G	Q/H	<u>Q/L*</u>	Y/V	Q/G	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G	Q/G	Q/G	
DD2	2003	Africa	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G	Q/G	Q/G	
US-PC0082	2010	USA	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G	Q/G	Q/G	

## 5.5 Discussion

Canine Kobuvirus (CaKoV) is an emerging pathogen in Thailand. To the best of our knowledge, the CaKoV was described in Asia in retrospective study in Korea in 2011 and have been reported in Japan, China, and Australia, respectively (Bodewes et al., 2014; Choi et al., 2015; Oem et al., 2014b; Soma et al., 2016b). However, the CaKoV have never been reported in the country or South East Asia region. In this study, during the 2 year-survey programs, we found CaKoV positivity at 17.59% in both sick (19.44%) and healthy (9.09%) animals. Compare to other studies, CaKoV % positivity in this study was lower than those in China (54%) and Korea (32.2%) (Li et al., 2018; Oem et al., 2014a). Our result showed that the CaKoV could be frequently detected in younger dogs at 27% which consistence with previous reports (Soma et al., 2016a). Similar to other previous studies, co-infections with other enteric viral pathogens were observed such as CaKoV/Canine parvovirus and CaKoV/Canine Coronavirus (Di Martino et al., 2013; Oem et al., 2014b; Soma et al., 2016a). Moreover, CaKoVs were detected in both diarrheic and non-diarrheic dogs which consistent with other studies (Oem et al., 2014b; Soma et al., 2016b). Our result supported that this virus may not be the only cause of enteric disease in dogs. Nevertheless, the CaKoV infection have still been identified in symptomatic dogs without other enteric pathogen infections (Di Martino et al., 2013). Our observation supported that the role of CaKoV as a primary pathogen of acute gastroenteritis remain unclear.

In this study, the genome size of 4 Thai CaKoVs is 7,530 bp with one ORF encoding 2,444 amino acids of a putative polyprotein, which comparable to previous reports. Genome organization of CaKoV includes leader protein (L), structural proteins (VP0, VP3, VP1), non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, 3D). Phylogenetic analyses showed that the Thai CaKoVs were closely related to each other and clustered with Aichivirus A. It is noted that Thai CaKoVs were closely related to Chinese CaKoVs sub-cluster but in separated sub-cluster from the viruses from the US, UK, Brazil, and Tanzania (Figure 5.3). Phylogenetic analyses of 3D gene showed similar result which Thai CaKoVs were grouped together with Chinese CaKoVs (G1 sub-cluster). This observation regarding to the sub-clusters of CaKoVs was in

agreement with the previous study (Li et al., 2016) . On the other hand, based on VP1 gene, the viruses can be clustered into 2 major subgroups, US/EU/Africa subgroup and China/Thailand subgroup which similar to the previous reports (Li et al., 2018; Olarte-Castillo et al., 2015) (Figure 5.4 and 5.5).

Genetic analyses of Thai CaKoVs showed that whole genome of 4 Thai CaKoVs posed highest nucleotide similarity to Chinese CaKoVs including SMCD-59 and CH-1. This observation supported phylogenetic analysis that Thai CaKoVs were closely related to Chinese CaKoVs sub-cluster but in separated sub-cluster from the viruses from the US, UK, Brazil, and Tanzania. Of all viral genes, the VP1 gene was the most diverse gene among Thai CaKoVs and other reference CaKoVs. Similar observation was also reported in previous study that VP1 protein is the most variable capsid protein (Chen et al., 2013). It is noted that the putative proline rich region at VP1-228-240 (P<sub>228</sub>XPPPPXPPXP<sub>240</sub>) was observed both in Thai CaKoVs and reference viruses. Previous studies indicated that proline rich region may associate with enteric receptor binding of the viruses (Chen et al., 2013; Oem et al., 2014a). It is noted that Thai CaKoVs posed unique PPP (VP1; 228-240), which also observed most reference viruses from China, Korea, Japan, US, UK suggesting unique characteristic. These unique amino acids were not observed in the CaKoV from the Australia (CE9), Brazil (BRA/26) and Tanzania (TZ/75, TZ82) (Olarte-Castillo et al., 2015; Ribeiro et al., 2017). However, the association of these unique amino acids and viral pathogenesis still needs to be further investigated. Based on genetic analysis, unique amino acids at the position, 65V, 67D, 199L, 138T, 150P, 153D, 201S, 204Q, 205Q, 210Q, 213T and 241E were observed. These unique amino acids of China/Thailand sub-cluster could be benefit for the detection of virus origin or diagnostic purpose in the future. Similar to previous study, analysis of predicted amino acid cleavage sites of whole genome were conserved among CaKoVs except one variation at 776/777 (VP3/VP1) which unique in wild carnivores (Olarte-Castillo et al., 2015).



## 5.6 Conclusion

In conclusion, this study is the first to report of canine Kobuvirus in dogs in Thailand. CaKoVs were mostly detected in clinical dogs of young age. However, the viruses could be detected from both healthy and sicked dogs. Genetic and phylogenetic analyses showed that whole genomes of Thai CaKoVs were closely related to Chinese CaKoVs in 2015 (SMCD-59) with high nucleotide similarity suggesting a possible origin of CaKoVs in Thailand. CaKoV is considered as an emerging viral pathogen in the domestic dogs. Since CaKoVs have never been reported in the country and SEA region, the detection and characterization of CaKoV from different parts of the regions should be extended for better understanding the epidemiology and evolution of CaKoVs. Our result raises the concerns to vet practitioners that diarrhea in dogs due to canine Kobuvirus infection should not be ignored.



## CHAPTER VI NOROVIRUS

Parts of this work have been published in

### Human Norovirus Infection in Dogs, Thailand

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#### 6.1 Abstract

In July 2018, recombinant norovirus GII.Pe-GII.4 Sydney was detected in dogs who had diarrhea in a kennel and in children living on the same premises in Thailand. Whole-genome sequencing and phylogenetic analysis of 4 noroviruses from Thailand showed that the canine norovirus was closely related to human norovirus GII.Pe-GII.4 Sydney, suggesting human-to-canine transmission.

## 6.2 Introduction

Norovirus (NoV) infection is a major cause of both endemic and epidemic acute gastroenteritis. NoVs have been classified into seven genogroups based on the VP1 major capsid protein. NoVs GI, GII and GIV can infect humans, NoVs GII can infect pigs, NoVs GIII and GV can infect ruminants and mice, and NoVs GVI and GVII can infect dogs (Vinje, 2015). The evolutionary mechanisms of NoVs can be analyzed based on the recombination at the RNA-dependent RNA polymerase (RdRp) (ORF1) and the major capsid protein VP1 (ORF2) loci of the norovirus genome (Zheng et al., 2006). Newly emerged norovirus strains may lead to increasing incidence of norovirus infection worldwide. GII.4 is one of the predominant globally circulating genotypes. Genetic diversity of NoVs has been reported in a wide range of animals, such as pigs, cattle, and dogs.

Canine norovirus (CaNoV) was first reported as GIV.2 genotype in Italy in 2007 (Martella et al., 2008). Subsequently, the CaNoVs have been reported to cause diseases in dogs in several countries in Asia and Europe (Caddy et al., 2013; Mesquita et al., 2010a; Mesquita and Nascimento, 2012; Ntafis et al., 2010). On the other hand, the seroprevalence of human noroviruses (HuNoVs) in dogs in the UK was reported as 13% (Caddy et al., 2013). Notably, GII.4 genotype (variants GII.4-2006b and GII.4-2008) was reported in dogs in Finland, indicating HuNoVs could transmit to and cause diarrhea in dogs (Summa et al., 2012). In humans, CaNoV antibodies were also reported in veterinarians, whose experience high risk exposure (Mesquita et al., 2013). However, there are only a few reports of HuNoV infection in dogs, and only limited numbers of complete CaNoV genomes are available in the GenBank database. This study reported the evidence of HuNoV infection in dogs and children from a dog kennel in Thailand.

## 6.3 Material and method

### 6.3.1. Infection in Dogs

During July-September 2018, the Center of Excellence for Emerging and Re-emerging Diseases in Animals at Chulalongkorn University (Bangkok, Thailand) investigated a suspected outbreak of norovirus infection in dogs that had fever, acute vomiting, and watery diarrhea in a small-scale dog kennel. Epidemiologic investigation, sample collection, and laboratory diagnosis were conducted to determine the cause of the outbreak. Information from the outbreak investigation showed that 2 weeks before reporting of cases in animals, 2 children (8 months and 2 years of age) who lived on the kennel premises had been hospitalized on July 18, 2018 because of vomiting and watery diarrhea. These children recovered within 1 week. During hospitalization, human cases were diagnosed and confirmed as norovirus infection by using a rapid test kit. Animal sample collection and testing were performed under the Chulalongkorn University Animal Care and Use Committee Protocol (Institutional Animal Care and Use Committee no. 1731074). Human sample collection and testing were performed at the Center of Excellence for Clinical Virology under the Institutional Review Board of Chulalongkorn University Hospital protocol for human study (Institutional Review Board no. 634/59).

### 6.3.2. Identification of Viruses

Over 4 visits during July-September 2018, we collected 75 samples: 4 stool samples from 2 children (8 months and 2 years of age) and 71 rectal swab samples from 18 adult dogs and 6 puppies. We identified noroviruses by using an RT-PCR specific for the RNA dependent RNA polymerase gene (Kojima et al., 2002; Phumpholsup et al., 2015b). Because dogs showed clinical signs similar to those for canine enteric diseases, all samples were also examined for canine parvovirus type 2, rotavirus A, canine coronavirus, and canine distemper to rule out other canine enteric diseases (Buonavoglia et al., 2001a; Frisk et al., 1999; Gouvea et al., 1990; Herrewegh et al., 1998; Mesquita et al., 2010b). We extracted virus RNAs from 10% stool suspensions in phosphate-buffered saline, pH 7.2, and from rectal swab samples by

using the QIAAsymphony DSP Viral/Pathogen Mini Kit (QIAGEN, <https://www.qiagen.com>) following the manufacturer's instructions. The virus RNA was stored at  $-80^{\circ}\text{C}$  until use.

A PCR for norovirus identification was conducted as described (Kojima et al., 2002; Phumpholsup et al., 2015b). We use a set of oligonucleotide primers (Table 6.1) A 1-step reverse transcription PCR (RT-PCR) (Invitrogen, <https://www.thermofisher.com>) was conducted in a final volume of 25  $\mu\text{L}$  containing 3  $\mu\text{L}$  of template RNA, 12.5  $\mu\text{L}$  of 2 $\times$  reaction mixture, 0.6  $\mu\text{L}$  of 10  $\mu\text{mol/L}$  of forward (F4895) and reverse (R5591) primers, 1.2  $\mu\text{L}$  of SuperScript III reverse transcriptase (Invitrogen), and distilled water. The RT-PCR procedure included a reverse transcription step at  $55^{\circ}\text{C}$  for 30 min; an initial denaturation step at  $94^{\circ}\text{C}$  for 2 min; followed by 40 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $50^{\circ}\text{C}$  for 30 s, and extension at  $68^{\circ}\text{C}$  for 1 min; and final extension step at  $68^{\circ}\text{C}$  for 6 min. To confirm the presence of noroviruses, 4  $\mu\text{L}$  PCR product was subjected to electrophoresis on a 1.5% agarose gel, with RedSafe dye (Bulldog Bio, <https://www.bulldog-bio.com>), at 100 V for 45 min. The amplification product was visualized on a UV transilluminator. The expected size of the norovirus-positive amplified product was 493 bp. We conducted a 1-step real-time RT-PCR for norovirus identification as described (Chuchaona et al., 2019; Debbink et al., 2013) This real-time RT-PCR was conducted by using the TaqMan Fast Virus 1-step real-time RTPCR (Thermo Fisher Scientific, <https://www.thermofisher.com>) with specific primers and probe to GI and GII noroviruses was conducted in a final volume of 25  $\mu\text{L}$  containing 5  $\mu\text{L}$  of template RNA, 1 $\times$  Master Mix, 0.25  $\mu\text{mol/L}$  GI forward and reverse primers, 0.125  $\mu\text{mol/L}$  of GI-JOE labeled probe, 0.25  $\mu\text{mol/L}$  GII forward and reverse primers, 0.125  $\mu\text{mol/L}$  of GII-FAM labeled probe, and distilled. This real-time RT-PCR included a reverse transcription step at  $50^{\circ}\text{C}$  for 10 min; an enzyme activation step at  $95^{\circ}\text{C}$  for 20 s; followed by 45 cycles of denaturation at  $95^{\circ}\text{C}$  for 3 s and annealing at  $60^{\circ}\text{C}$  for 30 s. A cycle threshold value  $<40$  was considered as indicating GI and GII positive.

**Table 6.1.** List of primers for identification and sequencing of noroviruses, Thailand

Primer	Sequence, 5'→3'	Position	Target	Reference
F4895	GATTTAGGTGACACTATAGYDSTT YTCHTTYTAYGGKAYGATGA	4585	RdRp	(Phumpholsup et al., 2015a)
R5591	AWTCGGGCARGAGATYGCATC	5078	RdRp	(Kojima et al., 2002)
G2SKR	CCRCCNGCATRHCCRTTRTACAT	5389	VP1	(Kojima et al., 2002)
NOV-ORF1-1F	GTGAATGAAGATGGCSTCTAACG	1	ORF1	This study
NOV-ORF1-1R	CCTGTTCCAATCCTGGTACG	705		This study
NOV-ORF1-2F	TCTCTCCAGACACTCTTAGG	572		This study
NOV-ORF1-2R	GCATCCTCGATGGAYCTCAC	1233		This study
NOV-ORF1-3F	TAGGTTTGGTGCTAGGATTAC	1065		This study
NOV-ORF1-3R	CCTTTGTTCTCAATTCTGTC	1740		This study
NOV-ORF1-4F	CAGCGYTRGGYCTTATCC	1580		This study
NOV-ORF1-4R	CTGACATRGCTTGACATCCTT	2208		This study
NOV-ORF1-5F	GAGCATCAGGGTTACTCCATG	2066		This study
NOV-ORF1-5R	CTCTTGACTCRTCGTACTCCTCAT	2700		This study
NOV-ORF1-6F	CACAGAAGAGATGGCCAACA	2561		This study
NOV-ORF1-6R	CTAGAATCATGCCCGTCACATC	3227		This study
NOV-ORF1-7F	CTGGTCGCGGATAGTCAACT	3062		This study
NOV-ORF1-7R	TTCTTTCCCTCTTCAAACATTAGG	4038		This study
NOV-ORF1-8F	TCAARGGTGGCCCTTCATTGC	3726		This study
NOV-ORF1-8R	AAGGGAGTTGGCCTGAATGAT	4561		This study
NOV-ORF1-9F	CAGAACCACACCTGGCCCAG	4371		This study
NOV-ORF1-9R	GTCAATTACATTTTTGTTGGCCCGC	5210		This study
NOV-ORF2-1F	AGACAAGAGCCAATGTTTCAG	5004	ORF2	This study
NOV-ORF2-1R	GTGCCTAGGAGCACGCCATCAG	5887		This study
NOV-ORF2-2F	TGAGGAGATGACCAATTCAAGA	5787		This study
NOV-ORF2-2R	ATCCAGCAAAGAAAGCTCCAGC	6709		This study
NOV-ORF3-1F	AGGTTTGATTCTGGGTYAACCAG	6630	ORF3	This study
NOV-ORF3-1R	CGTGACTCCCCYCGCTTACG	7487		This study
VN3T20	GAGTGACCGCGCCGCT20		Poly A	(He et al., 2016)

### 6.3.3. Characterization of norovirus

In this study, we selected 4 noroviruses from Thailand: including 2 from humans (CU21953 and CU21954) and 2 from dogs (CU21939 and CU21952) for whole-genome sequencing. Whole norovirus genomes were sequenced by using oligonucleotide primer sets previously described and new primer sets designed with Primer 3 Plus (Table 6.1) (He et al., 2016; Untergrasser et al., 2012). A 25  $\mu$ L RT-PCR mixture contained 3  $\mu$ L of template RNA, 12.5  $\mu$ L of 2 $\times$  reaction mixture, 0.6  $\mu$ L of 10  $\mu$ mol/L forward and reverse primers, 1.2  $\mu$ L of SuperScript III reverse transcriptase, and distilled water. The RT-PCR procedure included a reverse transcription step at 55°C for 30 min; an initial denaturation step at 94°C for 2 min; followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 48–55°C for 30 s, and extension at 68°C for 2 min; and a final extension step at 68°C for 6 min. Amplicons were gel-purified and sequenced (First Base Laboratories, <http://www.firstbaselab.com>). Nucleotide sequences were assembled and validated by using SeqMan software version 5.03 (DNASTAR Inc., <https://www.dnastar.com>). Whole-genome sequences of noroviruses from Thailand were submitted to GenBank under accession nos. MK928496–9.

In this study, we selected 4 Thai NoVs including 2 NoVs from human (CU-21953, CU-21954) and 2 NoVs from dogs (CU-21939, CU-21952) for whole genome sequencing.

For pairwise comparisons and genetic analysis of noroviruses from Thailand, we aligned nucleotide sequences and deduced amino acids of noroviruses with reference noroviruses from GenBank by using MEGA version 7.026 (<https://www.mega-software.net>) and MegAlign version 5.03 (DNASTAR Inc.) software. For phylogenetic analysis, we compared complete genome sequences of noroviruses from Thailand with those of reference noroviruses, including genogroups GI (n = 2), GII (n = 5), GIII (n = 3), GIV (n = 4), GV (n = 2), GVI (n = 2), and GVII (n = 2). We analyzed the partial open reading frame 1 of noroviruses from Thailand NoVs by comparison with reference GII noroviruses, including GII.P1 (n = 2; United States), GII.P4 (n = 25; Australia, Japan, Georgia, South Korea, the Netherlands, Taiwan, United Kingdom and United States), GII.P5 (n = 1; Japan), GII.P6 (n = 2; Japan and United States), GII.P7 (n = 5; Japan, the Netherlands, and United States), GII.P8 (n = 1; Japan), GII.P11 (n = 1; China), GII.P12 (n = 7; China, South Korea, and Japan), GII.P16 (n = 6; Germany, Japan,

Russia, and United States), GII.P17 (n = 1; Hong Kong), GII.P18 (n = 1; United States), GII.P20 (n = 1; Germany), GII.P22 (n = 2; Japan), GII.P21 (n = 2; Japan and the Netherlands), GII.Pc (n = 1; United States), GII.Pe (n = 10; Australia, China, Japan, and Thailand), GII.Pg (n = 2; Australia and China), and outer group GI.P1 (n = 1; United States). We compared the partial open reading frame 2 ORF2 of noroviruses from Thailand with those of reference of GII noroviruses, including genogroups GII.1 (n = 1; United States), GII.2 (n = 1; United Kingdom), GII.3 (n = 3; Argentina, Canada, and the Netherlands), GII.4 (n = 40; Australia, Canada, China, Finland, Ireland, Japan, Netherlands, Thailand, United Kingdom, and United States), GII.5 (n = 1; United Kingdom), GII.6 (n = 22; China, Japan, Italy, Taiwan, United Kingdom, and United States), GII.7 (n = 12; Japan, Netherlands, Germany, Italy, United Kingdom, and United States), GII.8 (n = 4; China, the Netherlands, and Russia), GII.9 (n = 1; United States), GII.10 (n = 1; Germany), GII.11 (n = 1; Japan), GII.12 (n = 1; United Kingdom), GII.13 (n = 18; China, Nepal, and United States), GII.14 (n = 14; Germany, Japan, and United States), GII.16 (n = 1; United States), GII.17 (n = 1; United States), GII.18 (n = 1; United States), GII.19 (n = 1; United States), GII.20 (n = 1; Germany), GII.21 (n = 18; Bhutan, China, Cambodia, Hong Kong, India, Iraq, Japan, South Korea, Russia, United Kingdom, and United States), GII.22 (n = 1; Japan), and outer groups; GI (n = 1; United States) and GVII (n = 1; Hong Kong). Phylogenetic analysis was performed using MEGA version 7.026 with the neighbor-joining algorithm and bootstrap analysis of 1,000 replications.

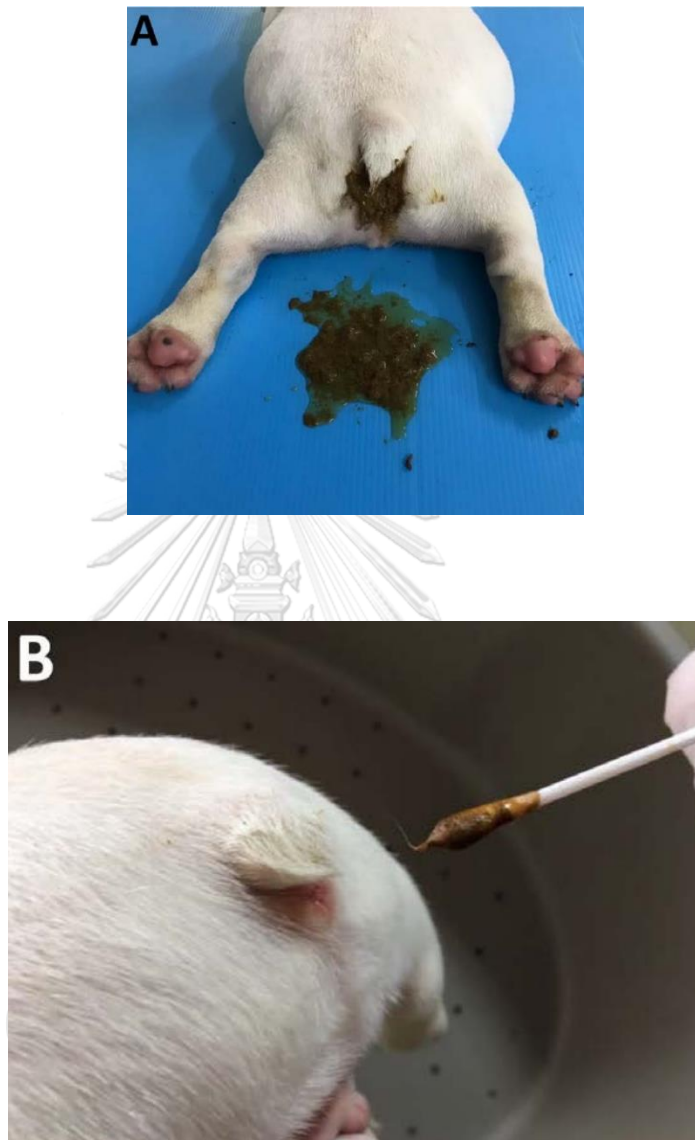


#### 6.4 Results and discussion

On July 27, 2018, we investigated acute gastroenteritis in dogs in a dog kennel. An outbreak occurred in a small-scale dog kennel that contained 18 adult dogs in Suphanburi, central Thailand. Clinical signs in bitches and puppies were fever, acute watery diarrhea, and mild dehydration (Figure 6.1). Information for the outbreak investigation indicated that 2 weeks earlier (July 18), 2 children (8 months and 2 years of age) who lived on the kennel premises were hospitalized because of vomiting and watery diarrhea. These children recovered within 1 week. During hospitalization, human cases were diagnosed and confirmed as norovirus infection by using a rapid test kit (RIDA QUICK Norovirus, [https:// clinical.r-biopharm.com](https://clinical.r-biopharm.com)). Five adults, 2 children, and 18 adult dogs were living on the premises. All dogs were housed in the kennel; only 2 apparently pregnant dogs (CU21939 and CU21952) were moved into the house of the owner. The 2 apparently pregnant dogs were kept in close contact with children.

On August 2, 2018, a pregnant dog gave birth to 6 puppies, and the other bitch was found to have a false pregnancy. During the 6 weeks (July 27–September 5) of the norovirus outbreak, 2 (11.11%) of 18 dogs (the 2 apparently pregnant dogs kept in the house of the owner) and 5 (83.33%) of 6 puppies showed clinical signs of infection (Table 6.2). After treatment and hygiene management, including separation of dogs, frequent cleaning, and disinfection, all dogs recovered, and no deaths occurred.

Animal samples were collected and examined at the Center of Excellence for Emerging and Re-emerging Infectious Diseases in Animals, Chulalongkorn University (Bangkok, Thailand). Studies were approved by the Institutional Animal Care and Use Committee (approval no. 1731074). Human samples were collected and submitted to the Center of Excellence for Clinical Virology under the Institutional review board of Chulalongkorn University (Institutional Review Board no. 634/59).



**Figure 6.1.** Human norovirus infection in dogs, Thailand. A) Diarrhea. B) Collection of fecal samples.

During the 4 visits in the study, we examined 75 samples (4 stool samples from 2 children, 71 rectal swab specimens from 18 adult dogs and 6 puppies). We detected norovirus by using a reverse transcription PCR specific for the RNA-dependent RNA polymerase gene as described (Kojima et al., 2002; Phumpholsup et al., 2015a). We detected norovirus in samples from children (4/4), adult dogs (2/53), and puppies (10/18) (Supplement Table 6.1). All human samples were positive for norovirus at the first (July 27) and third (August 25) visits. The 2 bitches with clinical signs were positive for norovirus at the first visit (July 27). Their puppies (5/6) were positive at the second (August 18) and third (August 25) visits. Our findings are consistent with a previous report that animals can shed noroviruses for a long period (Martella et al., 2008). All samples were also tested for canine parvovirus type 2, rotavirus A, canine coronavirus, and canine distemper virus to rule out other canine enteric diseases; all showed negative results (Table 6.2).

**Table 6.2** Characteristics of samples collected and examined from a dog kennel, Thailand, 2018\*\*

Sample name	Sample ID	Collection				Sample	Clinical sign	NoV RT-		NoV real-time		CaCoV	CDV
		date	Sex	Age	Breed			PCR	RT-PCR	CPV2	RVA		
<b>First visit, n = 19</b>													
Human 1	CU21953+	Jul 27	M	2 y	Not applicable	Feces	Soft stool	+	+ (27.3)	-	-	NA	NA
Human 2	CU21954+	Jul 27	M	8 mo	Not applicable	Feces	Soft stool	+	+ (20.5)	-	-	NA	NA
Dog 1	CU21936	Jul 27	F	6 mo	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	+	-
Dog 2	CU21937	Jul 27	M	6 mo	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 3	CU21938	Jul 27	F	2 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 4	CU21939+	Jul 27	F	1 y	French bulldog	Rectal swab	Watery diarrhea	+	+ (29.7)	-	-	-	-
Dog 5	CU21940	Jul 27	F	3 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 6	CU21941	Jul 27	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 7	CU21942	Jul 27	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	+	-
Dog 8	CU21943	Jul 27	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	+	-
Dog 9	CU21944	Jul 27	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	+	-
Dog 10	CU21945	Jul 27	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 11	CU21946	Jul 27	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	+	-
Dog 12	CU21947	Jul 27	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 13	CU21948	Jul 27	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 14	CU21949	Jul 27	M	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 15	CU21950	Jul 27	M	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 16	CU21951	Jul 27	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 17+	CU21952+	Jul 27	F	3 y	French bulldog	Rectal swab	Watery diarrhea	+	+ (29.6)	-	-	-	-
<b>Second visit, n = 24</b>													
Puppy 1§	CU22011	Aug 18	M	2 wk	French bulldog	Rectal swab	Watery diarrhea	+	+ (30.5)	-	-	-	-
Puppy 2	CU22012	Aug 18	M	2 wk	French bulldog	Rectal swab	Watery diarrhea	+	+ (30.1)	-	-	-	-
Puppy 3	CU22013	Aug 18	F	2 wk	French bulldog	Rectal swab	Watery diarrhea	+	+ (31.4)	-	-	-	-
Puppy 4	CU22014	Aug 18	F	2 wk	French bulldog	Rectal swab	Watery diarrhea	+	+ (30.7)	-	-	-	-
Puppy 5	CU22015	Aug 18	F	2 wk	French bulldog	Rectal swab	Watery diarrhea	+	+ (31.8)	-	-	-	-
Puppy 6	CU22016	Aug 18	F	2 wk	French bulldog	Rectal swab	Watery diarrhea	-	-	-	-	-	-
Dog 1	CU22020	Aug 18	F	6 mo	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 2	CU22019	Aug 18	M	6 mo	French bulldog	Rectal swab	Asymptomatic	-	+ (36.0)	-	-	-	-
Dog 3	CU22018	Aug 18	F	2 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 4	CU22034	Aug 18	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 5	CU22022	Aug 18	F	3 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 6	CU22026	Aug 18	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 7	CU22021	Aug 18	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 8	CU22025	Aug 18	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 9	CU22023	Aug 18	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 10	CU22029	Aug 18	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 11	CU22030	Aug 18	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 12	CU22024	Aug 18	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 13	CU22031	Aug 18	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 14	CU22028	Aug 18	M	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 15	CU22032	Aug 18	M	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 16	CU22027	Aug 18	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	+ (37.0)	-	-	-	-
Dog 17+	CU22033	Aug 18	F	3 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-
Dog 18	CU22017	Aug 18	F	5 y	Miniature pinscher	Rectal swab	Asymptomatic	-	-	-	-	-	-
<b>Third visit, n = 9</b>													
Human 1	CU22080	Aug 25	M	2 y	Not applicable	Feces	Asymptomatic	+	S (40.0)	-	-	NA	NA
Human 2	CU22081	Aug 25	M	8 mo	Not applicable	Feces	Asymptomatic	+	+ (33.4)	-	-	NA	NA
Puppy 1	CU22072	Aug 25	M	3 wk	French bulldog	Rectal swab	Watery diarrhea	+	+ (32.3)	-	-	-	-
Puppy 2	CU22073	Aug 25	M	3 wk	French bulldog	Rectal swab	Watery diarrhea	+	+ (33.2)	-	-	-	-
Puppy 3	CU22074	Aug 25	F	3 wk	French bulldog	Rectal swab	Watery diarrhea	+	+ (31.9)	-	-	-	-
Puppy 4	CU22075	Aug 25	F	3 wk	French bulldog	Rectal swab	Watery diarrhea	+	+ (32.5)	-	-	-	-
Puppy 5	CU22076	Aug 25	F	3 wk	French bulldog	Rectal swab	Watery diarrhea	+	+ (32.5)	-	-	-	-
Puppy 6	CU22078	Aug 25	F	3 wk	French bulldog	Rectal swab	Watery diarrhea	-	-	-	-	-	-
Dog 17*	CU22079	Aug 25	F	3 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-

**Table 6.2.** Characteristics of samples collected and examined from a dog kennel, Thailand, 2018\*\* (cont.)

Sample name	Sample ID	Collection				Breed	Sample	Clinical sign	NoV RT-		NoV real-time			
		date	Sex	Age					PCR	RT-PCR	CPV2	RVA	CaCoV	CDV
<b>Fourth visit, n = 23</b>														
Puppy 1§	CU22143	Sep 5	M	1 mo	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-	
Puppy 2	CU22144	Sep 5	M	1 mo	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-	
Puppy 3	CU22145	Sep 5	F	1 mo	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-	
Puppy 4	CU22146	Sep 5	F	1 mo	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-	
Puppy 5	CU22147	Sep 5	F	1 mo	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-	
Puppy 6	CU22148	Sep 5	F	1 mo	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-	
Dog 1	CU22151	Sep 5	F	7 mo	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-	
Dog 2	CU22150	Sep 5	M	7 mo	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-	
Dog 3	CU22153	Sep 5	F	2 y	French bulldog	Rectal swab	Asymptomatic	-	NA	-	-	-	-	
Dog 5	CU22155	Sep 5	F	3 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-	
Dog 6	CU22161	Sep 5	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	NA	-	-	-	-	
Dog 7	CU22156	Sep 5	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-	
Dog 8	CU22152	Sep 5	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-	
Dog 9	CU22157	Sep 5	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-	
Dog 10	CU22154	Sep 5	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-	
Dog 11	CU22158	Sep 5	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	-	-	-	-	-	
Dog 12	CU22163	Sep 5	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	NA	-	-	-	-	
Dog 13	CU22164	Sep 5	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	NA	-	-	-	-	
Dog 14	CU22149	Sep 5	M	1 y	French bulldog	Rectal swab	Asymptomatic	-	NA	-	-	-	-	
Dog 15	CU22159	Sep 5	M	1 y	French bulldog	Rectal swab	Asymptomatic	-	NA	-	-	-	-	
Dog 16	CU22162	Sep 5	F	1 y	French bulldog	Rectal swab	Asymptomatic	-	NA	-	-	-	-	
Dog 17‡	CU22160	Sep 5	F	3 y	French bulldog	Rectal swab	Asymptomatic	-	NA	-	-	-	-	
Dog 18	CU22165	Sep 5	F	5 y	Miniature pinscher	Rectal swab	Asymptomatic	-	NA	-	-	-	-	

\*Numbers in parentheses are cycle threshold values. CaCoV, canine coronavirus; CDV, canine distemper virus; CPV2, canine parvovirus 2; ID, identification; NA, not available; NoV, norovirus; RT-PCR, reverse transcription PCR; RVA, rotavirus A; +, positive; -, negative.

†Samples were subjected to whole-genome sequencing.

‡Dog 17 was a bitch with 6 puppies.

§Puppies 1–6 were from the same litter of dog 17.

We selected 4 of the noroviruses, 2 from humans (CU21953 and CU21954) and 2 from dogs (CU21939 and CU21952), for whole-genome sequencing by using oligonucleotide primer sets (Table 6.1). We then submitted nucleotide sequences for these viruses (GenBank accession nos. MK928496–9) (Table 6.3). Phylogenetic analysis showed that the noroviruses in this investigation clustered in genotype GII.4. In general, canine noroviruses are commonly grouped into genogroups GIV, GVI, and GVII. In contrast, noroviruses from these dogs were closely related to human noroviruses and viruses in genogroup GII (Figure 6.2). Phylogenetic analysis of partial open reading frame 1 (ORF1) and ORF2 showed that all noroviruses from this investigation clustered with norovirus GII.Pe-GII.4 Sydney 2012, which were reported to be circulating worldwide (Figure 6.3 and Figure 6.4) (Cannon et al., 2017). Noroviruses from dogs in this study (GII.4 Sydney) were in different clusters from canine noroviruses 3–09 (GII.4 Den Haag) and 261–10 and 1C-09 (GII.4 unclassified) reported in Finland (Summa et al., 2012).

We compared nucleotide and deduced amino acids of the noroviruses from this investigation with reference canine and human noroviruses. On the basis of antigenic epitopes (A–E) of major capsid protein that correlate with blockade of neutralization antibodies, the noroviruses from Thailand had specific amino acids in specific positions consistent with those for human norovirus GII.Pe-GII.4 Sydney, which were not observed in human norovirus genogroups GI and GIV and canine norovirus genogroups GIV and GVII (Table 6.4).

Pairwise comparisons of whole-genome sequences showed that the viruses had 99.90% nt identities (only 3 nt differences in ORF2; T1176C [silent mutation 392G], C1354T [silent mutation 452L] and in ORF3; T803A [V268E]) to each other and highest nucleotide identities to human norovirus from China [99.00%; JN010] and the human norovirus reference Sydney strain [97.6%; NSW0514]). On the basis of partial ORF2 sequences, we showed that the canine noroviruses from this investigation were different from canine noroviruses GII.4 (3-09, 1C-09, and 261-10; 91.6% nt identities) and GIV, GVI, and GVII (52.90%–55.50% nt identities) (Table 6.5).

**Table 6.3.** Detail description of Thai NoVs characterized in this study.

Virus	Host	Sample	Month/Year	Age	Sequences	GenBank Accession No.
GII/Hu/THA/2018/GII.Pe-GII.4/CU21953	Human	Feces	July/18	2 years	WG	MK928496
GII/Hu/THA/2018/GII.Pe-GII.4/CU21954	Human	Feces	July/18	8 months	WG	MK928497
GII/Hu/THA/2018/GII.Pe-GII.4/CU21939	Dog	Rectal swab	July/18	2 years	WG	MK928498
GII/Hu/THA/2018/GII.Pe-GII.4/CU21952	Dog	Rectal swab	July/18	3 years	WG	MK928499

\*WG: whole genome sequences



**Table 6.4.** Genetic analysis of nucleotide sequences of canine and human noroviruses from Thailand for antigenic epitopes (A–E) major capsid protein compared with those for other noroviruses\*

Virus	County/year	Accession no.	Variant	Antigenic epitopes																
				A					B					C					D	
				294	296	297	298	368	372	373	382	382	340	376	393	394	395	407	412	413
<b>Human</b>																				
Lordsdale	UK/1993	X86557	Bristol 1993	A	S	H	D	T	N	L	K	K	A	Q	D	-	H	N	T	G
Cambenwell	AU/1994	AF145896	Cambenwell 1994	V	S	H	D	T	N	L	K	K	A	Q	D	-	H	N	T	G
Farmington Hills	USA/ 2002	AY502023	Farmington Hills 2002	A	T	H	N	N	N	M	K	K	G	E	N	G	T	S	T	G
Hunter504D/04O	AU/2004	DQ078814	Hunter 2004	A	T	Q	N	S	S	V	R	R	R	E	S	T	T	D	D	S
CGMH09	TW/2006	JN400607	Den Haag 2006b	A	S	R	N	S	E	V	K	K	G	E	S	T	T	S	N	V
JB-15	KOR/2015	HQ009513	Apeldoorn 2008	T	S	R	N	A	D	V	K	K	A	D	N	T	A	S	N	S
New Orleans1805	USA/2009	GU445325	New Orleans 2009	P	S	R	N	A	D	V	K	K	T	E	S	T	T	S	N	I
NSW0514	AU/2012	JX459908	Sydney 2012	T	S	R	N	E	D	V	K	K	T	E	G	T	T	S	N	T
JN010	CHN/2017	MG214988	Sydney 2012	T	S	H	N	E	N	M	K	K	T	E	G	T	T	S	N	T
DBM15-156	THA/2015	MG786781	Sydney 2012	T	S	R	N	E	D	M	K	K	T	E	S	T	T	S	N	T
HuNoV/CU21953	THA/2018	This study	Sydney 2012	T	S	H	N	E	N	M	K	K	T	E	G	T	T	S	N	T
HuNoV/CU21954	THA/2018	This study	Sydney 2012	T	S	H	N	E	N	M	K	K	T	E	G	T	T	S	N	T
<b>Canine</b>																				
CaNoV/CU21952	THA/2018	This study	Sydney 2012	T	S	H	N	E	N	M	K	K	T	E	G	T	T	S	N	T
CaNoV/CU21939	THA/2018	This study	Sydney 2012	T	S	H	N	E	N	M	K	K	T	E	G	T	T	S	N	T

\*AU, Australia; CaNoV, canine norovirus; HuNoV, human norovirus; KOR, South Korea; THA, Thailand; TW, Taiwan.



**Table 6.5.** Pairwise comparisons of nucleotides and amino acids of canine norovirus CU21939 from Thailand with those of reference noroviruses\*

Virus	Genotype		Accession no.#	Variant†	Nucleotide (amino acid) identity, %				
	Host	†			Country/year	WGS 1–7564‡	ORF1 5–5104‡	ORF2 5085–6707‡	ORF3 6707–7513‡
Canine									
AN843	Dog	GIV.2	USA/2011	MK067289	NA	NA	62.20 (47.80)§	55.30 (38.80)	50.90 (41.40)
170/07	Dog	GIV.2	Italy/2007	EU224456	NA	NA	64.50 (71.20)¶	54.20 (36.50)	51.40 (42.90)
AN1610	Dog	GIV.2	USA/2017	MK067288	NA	NA	62.30 (47.8)§	55.20 (38.10)	51.10 (42.00)
AN1663	Dog	GIV.2	USA/2017	MK067291	NA	NA	62.30 (47.8)§	55.10 (38.30)	51.20 (41.70)
AN1638	Dog	GIV.2	USA/2017	MK067290	NA	NA	62.60 (48.40)§	55.10 (38.30)	51.20 (41.70)
C33/Visou	Dog	GVI.2	Portugal/2007	GQ443611	NA	NA	64.90 (72.10)	53.90 (39.10)	53.90 (46.50)
FD53	Dog	GVI.2	UK/2007	JF930689	NA	NA	64.20 (71.20)¶	54.40 (39.10)	54.20 (46.50)
FD210	Dog	GVI.1	Italy /2007	JF939046	NA	NA	65.10 (70.80)¶	54.30 (38.60)	54.20 (44.10)
AN1633	Dog	GVI.1	USA/2017	MK067293	NA	NA	62.60 (48.40)§	55.50 (40.60)	53.10 (43.50)
AN1632	Dog	GVI.1	USA/2017	MK067292	NA	NA	62.40 (47.80)§	55.50 (40.60)	53.10 (43.50)
ITA/91	Dog	GVI.1	Italy /2007	FJ875027	NA	NA	65.10 (71.20)¶	55.00 (39.90)	53.40 (43.80)
63.15	Dog	GVI.2	Italy /2015	KY486329	NA	NA	65.10 (72.10)¶	54.20 (38.80)	55.20 (46.20)
AN1640	Dog	GVI.2	USA/2017	MK067295	NA	NA	62.40 (47.80)§	54.20 (38.90)	54.5 (44.70)
HKU Ca026F	Dog	GVII	China/2007	FJ692500	NA	58.50 (47.20)	62.20 (55.00)	52.90 (37.90)	43.80 (33.00)
HKU Ca035F	Dog	GVII	China/2007	FJ692501	NA	58.50 (47.30)	62.20 (55.00)	52.90 (38.10)	43.80 (33.00)
1C-09	Dog	GII.4	Finland/2009	JF746890	Unclassified	NA	NA	91.60 (91.60)**	NA
261-10	Dog	GII.4	Finland /2010	JF746891	Unclassified	NA	NA	91.60 (91.60)**	NA
3-09	Dog	GII.4	Finland /2009	JF746892	Den Haag 2006b	NA	NA	91.60 (97.40)**	NA
Human									
HuNoV/OC07138	Human	GII.Pe-GII.4	Japan/2007	AB434770	Osaka 2007	NA	94.80 (98.50)#	89.60 (94.60)	99.00 (98.90)
HuNoV/NSW001P	Human	GII.Pe-GII.4	USA/2008	GQ845367	New Orleans	89.10 (94.50)	94.50 (86.50)	94.10 (93.90)	93.60 (96.30)
HuNoV/New	Human	GII.P4-GII.4	USA/2009	GU445325	New Orleans	89.00 (94.70)	94.70 (86.70)	94.30 (93.70)	93.7 (96.10)
Orleans									
HuNoV/NSW0514	Human	GII.Pe-GII.4	Australia/2012	JX459908	Sydney 2012	97.6 (98.70)	98.70 (97.70)	99.20 (97.40)	97.00 (98.00)
HuNoV/CUHK363	Human	GII.Pe-GII.4	China/2012	KC175323	Sydney 2012	98.20 (99.20)	99.20 (98.20)	99.50 (98.10)	98.00 (98.50)
0									
HuNoV/JN010	Human	GII.Pe-GII.4	China/2017	MG214988	Sydney 2012	99.00 (99.50)	99.50 (99.00)	99.60 (99.00)	98.90 (99.4)
HuNoV/DBM15-	Human	GII.Pe-GII.4	Thailand/2015	MG786781	Sydney 2012	97.40 (98.80)	97.50 (99.50)§	97.50 (98.50)	95.90 (95.20)
156									
HuNoV /CU21953	Human	GII.Pe-GII.4	Thailand/2018	This study	Sydney 2012	99.90 (99.80)	99.80 (100)	99.90 (100.00)	99.90 (99.80)
HuNoV /CU21954	Human	GII.Pe-GII.4	Thailand/2018	This study	Sydney 2012	99.90 (99.80)	99.80 (100)	99.90 (100.00)	99.90 (99.80)
CaNoV/CU21952	Dog	GII.Pe-GII.4	Thailand/2018	This study	Sydney 2012	99.90 (99.80)	99.80 (100)	99.80 (99.80)	99.9 (99.60)
CaNoV/CU21939	Dog	GII.Pe-GII.4	Thailand/2018	This study	Sydney 2012	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)

\*CaNoV, canine norovirus; HuNoV, human norovirus; NA, not available; ORF, open reading frame; WGS, whole-genome sequencing.

†Genotype classification by the Norovirus Genotype Tool (<https://www.rivm.nl/mpf/typingtool/norovir>).

#Norovirus strain NSW0514 (JX459908) was used as a reference. Values are basepairs.

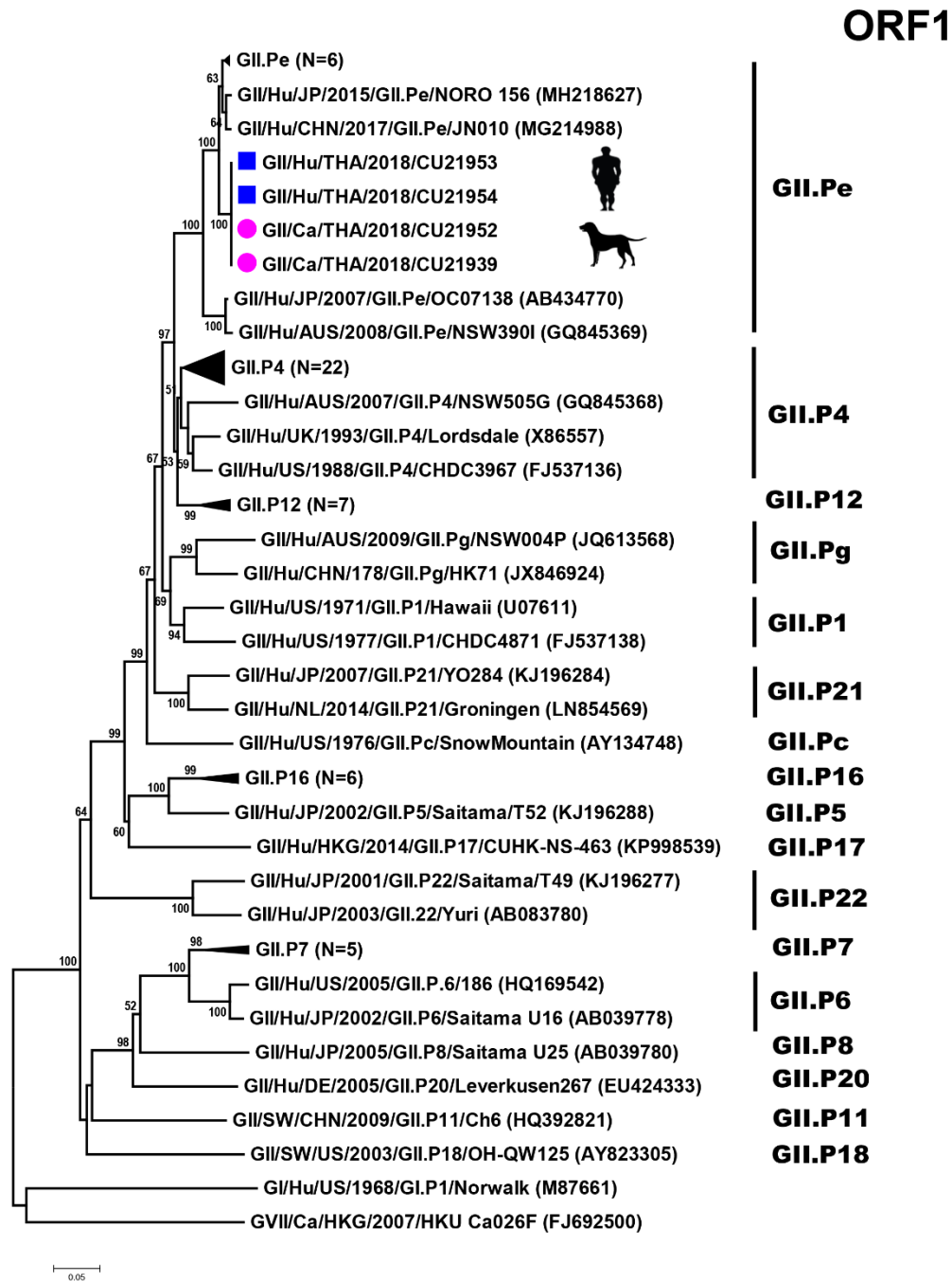
§Size of the ORF1 gene for genetic comparison is 5,088 bp.

¶Size of the ORF1 gene for genetic comparison is 699 bp.

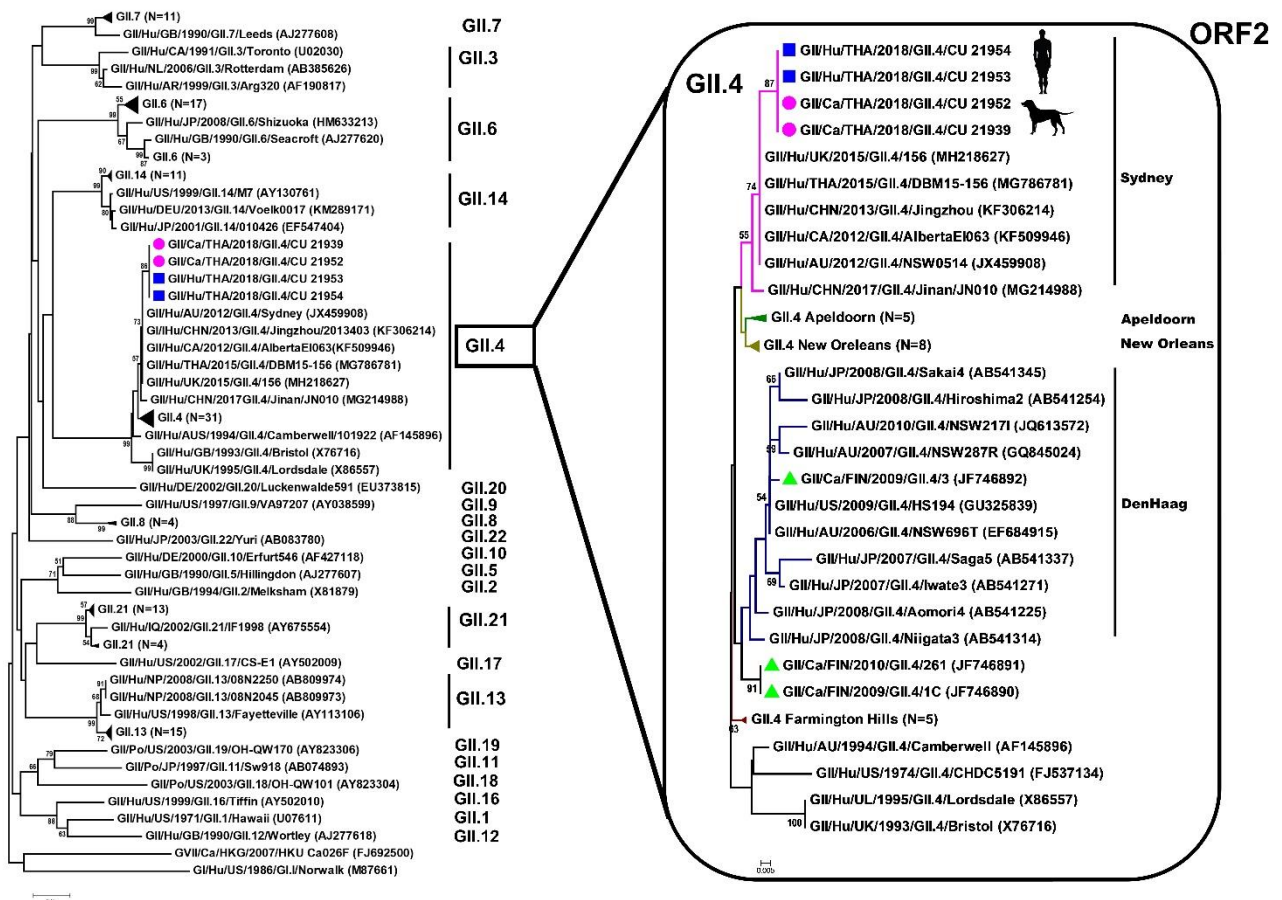
#Size of the ORF1 gene for genetic comparison is 805 bp.

\*\*Size of the ORF2 gene for genetic comparison is 228 bp.





**Figure 6.3.** Phylogenetic tree of open reading frame 1 of canine noroviruses (purple dots) and human noroviruses (blue squares) from Thailand and reference sequences. Tree was constructed by using MEGA version 7.026 (<https://www.megasoftware.net>) with the neighbor-joining algorithm and bootstrap analysis with 1,000 replications. Numbers along branches are bootstrap values, and numbers on the right indicate genogroups. Scale bar indicates nucleotide substitutions per site



**Figure 6.4.** A) Phylogenetic tree of ORF2 of noroviruses. B) Phylogenetic tree of ORF2 of GII.4 noroviruses. Red circles indicate canine noroviruses from Thailand, green triangles indicate canine noroviruses from Finland, and blue squares indicate human noroviruses from Thailand. Trees were constructed by using MEGA version 7.026 (<https://www.megasoftware.net>) with the neighbor-joining algorithm and bootstrap analysis with 1,000 replications. Numbers along branches are bootstrap values, and numbers on the right of panel A indicate genogroups. Scale bars indicate nucleotide substitutions per site. ORF, open reading frame.

## 6.5 Conclusion

We report infection of dogs with human norovirus GII.4 Sydney. Human noroviruses have been reported in dogs in Finland (GII.4 Den Haag and GII.4 unclassified) (Summa et al., 2012). Dogs showed mild clinical signs of acute watery diarrhea, similar to that for human norovirus infection, and low levels of illness and death. Similar observations have also been reported in other studies (Mesquita et al., 2013; Robilotti et al., 2015). In this study, children had been hospitalized 2 weeks before the investigation. Disease developed in dogs and puppies after they shared the same premises and possible direct contact with the children. This observation suggests potential human-to-dog transmission of human noroviruses. Genetic and phylogenetic analyses confirmed that whole genomes of canine and human noroviruses were closely related to human norovirus GII.Pe-GII.4 Sydney, suggesting that a common strain is circulating in Thailand and worldwide (Chuchaona et al., 2019; Kumthip et al., 2018). However, in our study, it is not clear how and when the viruses were introduced to children and dogs. In summary, we demonstrated evidence of norovirus GII.Pe-GII.4 infection in humans and dogs in Thailand. Dog owners and veterinarians should pay more attention to norovirus infection as a potential zoonotic and reverse zoonotic disease in households, animal hospitals, and shelters. Expanded surveillance for norovirus is needed to determine its status and distribution in human and dog populations.

## CHAPTER VII

### Canine parvovirus type 2c

Parts of this work have been published in

#### **Emergence of canine parvovirus type 2c in domestic dogs and cats from Thailand**

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#### **7.1 Abstract**

Canine parvovirus type 2 (CPV-2) is an important pathogen causing hemorrhagic enteritis in domestic dogs and wildlife worldwide. In early 2000, canine parvovirus type 2c (CPV-2c) was first reported and subsequently became a predominant subtype circulating in Europe and the Americas. CPV-2c has also been reported in Asia, including cases in China, India, Taiwan, and Vietnam. However, CPV-2c has never been reported in Thailand. In this study, we conducted viral enteric disease surveillance in dogs and cats in Thailand during 2016–2018. During 20 months of surveillance, 507 rectal swab samples were collected from dogs (n = 444) and cats (n = 63) with and without clinical signs. The samples were examined for parvovirus by using VP2 gene specific PCR for parvovirus. Our results showed that the positivity of canine parvovirus (CPV) was 29.95% and that of feline parvovirus (FPV) was 58.73%. In this study, we characterized 34 parvoviruses by VP2 gene sequencing. Moreover, two Thai-CPV-2 (Dog/CU-24 and Cat/CU-21) were characterized by whole genome sequencing. The phylogenetic results showed that Thai-CPV-2 had the highest nucleotide identities and clustered with Asian-CPV-2c but were in separate subclusters from the North American and European CPV-2c. Similarly, whole genome analyses showed that Thai-CPVs are closely related to Asian-CPV-2c, with unique amino acids at positions 297A, 324I, 370R and 426E. In summary, our results

demonstrated the emergence of Asian-CPV-2c in dogs and cats in Thailand. Thus, the surveillance of CPV-2 in domestic dogs and cats should be further conducted on a larger scale to determine the dynamics of predominant variants and their distributions in the country and in the Southeast Asia region.

**Keywords:** Canine Parvovirus, Characterization, Detection, Emergence, Thailand



## 7.2 Introduction

Canine parvovirus type2 (CPV-2) is an important pathogen for domestic dogs and wildlife worldwide. CPV-2, a non-envelop, single stranded DNA virus, belongs to the family *Parvoviridae*. CPV-2 causes acute hemorrhagic enteritis and myocarditis in dogs with high morbidity and frequent mortality (ranging 10-90%). In 1977, it was first reported that CPV-2 arose from feline panleukopenia virus (FPV) with at least six coding nucleotide differences in the VP2 gene. CPV-2 can be further grouped into three antigenic variants, including CPV-2a, CPV-2b and CPV-2c, based on unique amino acid residues at the positions 297 and 426 of VP2 (Buonavoglia et al., 2001b). CPV-2a and CPV-2b were reported in 1979 and 1984, with unique amino acid residues as 426N and 426D, respectively. Both CPV-2a and CPV-2b variants are distributed worldwide and infect both dogs and cats but exhibit low pathogenicity in cats (Clegg et al., 2012). In 1990, CPV-2a and CPV-2b were replaced by two new variants of CPV-2a (CPV-2a-297A) and CPV-2b (CPV-2b-297A), with one unique amino acid substitution, S297A (Decaro et al., 2009). In 2000, CPV-2c was first reported in Italy with one substitution at the VP2 gene (D426E) (Buonavoglia et al., 2001b). Recently, CPV-2c has been circulating predominantly in Europe and the Americas (Decaro and Buonavoglia, 2012). CPV-2c has also been reported in Asia, including cases in China, India, Taiwan and Vietnam (Chiang et al., 2016; Nakamura et al., 2004; Nandi et al., 2010; Zhao et al., 2016). It has also been reported that CPV-2c can cause severe diseases in cats (Miranda et al., 2014; Nakamura et al., 2001). In Thailand, CPV-2a and CPV-2b have been reported as major variants circulating in dogs (Phromnoi et al., 2010), while CPV-2c has never been reported in the country. In this study, CPV-2c was detected in domestic dogs and cats during a viral enteric disease surveillance. This study is the first to report and characterize an emergence of Asian-CPV-2c in domestic dogs and cats in Thailand.



### 7.3 Material and methods

During September 2016 to April 2018, the center of excellence for emerging and re-emerging infectious diseases in animals (CUEIDAs), Chulalongkorn University, conducted viral enteric disease surveillance in domestic dogs and cats in Thailand. The surveillance was carried out in four provinces of Thailand under the animal use and care protocol # 1731074. Rectal swab samples were mainly collected from dogs and cats with acute hemorrhagic or watery diarrhea, vomiting, fever, and dehydration. During 20 months of a surveillance, 507 rectal swab samples were collected from dogs (n=444) and cats (n=63) of young age (< 1 year), adult (1-5 years) and older (>5 years) with vaccination history record. Of 444 canine samples, 366 samples from sick dogs and 78 from healthy dogs were collected. Of 63 feline samples, 60 samples from sick cats and three from healthy animals were collected. All samples were subjected to parvovirus identification by PCR specific to VP2 gene as previously described (Buonavoglia et al., 2001b).

For parvovirus identification, viral DNA was extracted from rectal swab samples by using the QIA Symphony DSP viral/Pathogen mini kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The viral DNA was stored at  $-20^{\circ}\text{C}$  until used. PCR assay for parvovirus identification was conducted as previously described (Buonavoglia et al., 2001b). The oligonucleotide primers specific to VP2 gene were Hfor: 5'-CAGGTGATGAATTGCTACA-3' and Hrev: 5'-CATTGGATAAACTGGTGGT-3', located at position 3556-3575 and 4166-4185 of CPV-2, respectively. In brief, PCR was performed in a final volume of 20  $\mu\text{l}$  comprising 1  $\mu\text{l}$  of DNA, 0.8  $\mu\text{M}$  of each forward and reverse primer, 1 $\times$  TopTaq Master Mix (Qiagen, Hilden, Germany), 1 $\times$  Coral Load, and distilled water. The PCR condition was set as initial denaturation step at  $94^{\circ}\text{C}$  for 3 min 40 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $50^{\circ}\text{C}$  for 45 s and extension at  $72^{\circ}\text{C}$  for 1 min and final extension at  $72^{\circ}\text{C}$  for 7 min. The expected size of parvovirus positive amplified product was 611 bp. Identification of CPV2 antigenic variants was performed by using PCR-RFLP to differentiate CPV-2c and CPV-2a/CPV-2b variants. The PCR product size was 583 bp of the coding capsid protein VP2. Then, the PCR product was digested with enzyme

Mbo II (New England Biolabs, USA) that selectively recognizes the restriction site “GAAGA” (nucleotide 4062–4066 of the VP2 encoding gene). The CPV-2c was digested into two fragments of 500 bp and 83 bp (Buonavoglia et al., 2001b). The negative samples from CPV-2c PCR-RFLP assay were detected for CPV-2a and CPV-2b variants with specific primers (CPV-2abF/ CPV-2abR and CPV-2bF/CPV-2bR) generating the product size of 681 bp and 427 bp, respectively (Pereira et al., 2007; Pereira et al., 2000) (Table 7.1). Concurrently, the CPV-2a/CPV-2b samples were confirmed by sequencing of the flanking region at amino acid position 426 to identify CPV-2a or CPV-2b variants.

For parvovirus characterization, we selected two parvoviruses (Dog/CU-24 and Cat/CU-21) for whole genome sequencing and the other 32 parvoviruses (CPV-2 = 21, FPV = 11) for VP2 gene sequencing. The criteria for selecting these 34 viruses for genetic characterization were based on epidemiological and demographic data such as age of dog, date of isolation, breed, and vaccination history. The selection criteria for two viruses for whole genome sequencing were based on the representative of CPV-2c from dog (CU-24) and cat (CU-21). Parvovirus genome sequencing was conducted by using oligonucleotide primer sets previously described or new primer sets designed by using Primer 3 plus program (Table 7.1) (Buonavoglia et al., 2001b; Koressaar and Remm, 2007a; Untergasser et al., 2012). In brief, PCR was performed in a final volume of 30  $\mu$ l comprising 2  $\mu$ l of DNA, 0.4  $\mu$ M of each forward and reverse primer, 1x TopTaq Master Mix, 1x Coral Load, and distilled water. The PCR condition was set as initial denaturation at 94°C for 3 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 45 s, extension at 72°C for 2 min and final extension at 72°C for 7 min. PCR products were then purified and sequenced (1st Base Laboratories Sdn. Bhd., Malaysia). Nucleotide sequences were assembled by using SeqMan software v.5.03 (DNASTAR Inc., Madison, WI).

For genetic analysis, pairwise comparison was conducted by using MegAlign software v.5.03 (DNASTAR Inc., Madison, WI, USA). In brief, the nucleotide sequences and deduced amino acids of Thai-CPV-2 and FPV were aligned with those of vaccine

and reference strains of CPV2-a, CPV-2b, CPV-2c, CPV-2a-297A, CPV-2b-297A from USA (CPV-13/1981, CPV-411b/1998, OH20219/2015), Japan (Y1), China (SC-02/2011), India (KolkataD5/2014), Indonesia (HCM14/2013), Italy (288-01/2001, 1-99/1999), Vietnam (HCM7/2013) and Thailand (KU14/2008). Genetic analysis for CPV-2 antigen typing (VP2 at position 297 and 426) and important amino acid determinants (VP2 at position 300, 305, 321, 323, 324, 370, 371, 375) was conducted by alignment of VP2 gene by using MEGA v6.06 and MegAlign software v.5.03 (DNASTAR Inc., Madison, WI, USA). For phylogenetic analysis, the partial VP2 gene sequences of Thai-CPV-2 and FPV were analyzed with those of reference viruses. Vaccine and reference viruses including CPV-2-Vaccine strains (n=3), CPV-2a (n=2), CPV-2b (n=3), CPV-2c (n=14), CPV-2a-297A (n=11), CPV-2b-297A (n=7), FPV-Vaccine (n=3), FPV-G1 (n=9), FPV-G2 (n=1), and FPV-G3 (n=3) were included in the phylogenetic analysis. The maximum clade credibility (MCC) tree of partial VP2 gene was constructed by BEAST 1.8 with the Bayesian Markov Chain Monte Carlo (BMCMC) algorithm. A strict clock model with coalescent constant population and HKY with gamma 4 substitution were used as model parameters (Drummond et al., 2012a). The Bayesian MCMC chain lengths were 10,000,000 generations, with sampling every 10,000 generations. The tree iteration was discharged with 10% of the chains as burn in pattern by using a tree annotator, and the resulting MCC tree was drawn with Figtree software (v1.4.2) (Molecular evolution, phylogenetics and epidemiology, Edinburgh, Scotland, UK) (Figure 7.1). To determine the selective pressure on the partial VP2 (nucleotide positions 817–1314, amino acid positions 274–428), the ratio of nonsynonymous (dN) to synonymous (dS) substitutions was estimated using Mixed Effects Model of Evolution (MEME) within the HyPhy software package (Murrell et al., 2012). The significance levels were set at  $p = 0.1$ . The values  $dN/dS > 1$ ,  $dN/dS = 1$  and  $dN/dS < 1$  were used to define positive selection, neutral mutations, and negative selection, respectively. A phylogenetic tree was also constructed by using maximum likelihood with bootstrap

analysis of 1,000 replications using the MEGA v.6.06 program (Tamura et al., 2007) (Figure 7.2).



**Table 7.1.** List of primers for CPV2 typing and whole genome sequencing.

Primer name	Sequence (5'-3')	Position	Product size (bp)	Temp.	Reference
Hfor	CAG GTG ATG AAT TGC TAC A	3556-3575	611	50	Buonavoglia et al., 2001
Hrev	CAT TTG GAT AAA CTG GTG GT	4166-4185			
CPV 555F*	AGGAAGATATCCAGAAGGA	4003-4022	583	50	Buonavoglia et al., 2001
CPV 555 R*	GGTGCTAGTTGATATGTAATAACA	4585-4561			
CPV 2ab F**	GAAGAGTGGTTGTAATAATT	3025-3045	681	55	(Pereira et al., 2000)
CPV 2ab R**	CCTATATAACCAAAGTTAGTAC	3685-3706			
CPV 2b F***	CTTTAACCTTCCTGTAACAG	4043-4062	427	55	(Pereira et al., 2000)
CPV 2b R***	CATAGTTAAATTGGTTATCTAC	4470-4449			
CPV2_1F	TGA TAG GCG GTT TGT GTG TT	120-139	1010	50	This study
CPV2_1R	CAT TTG ATT GAC ACT TCC TTT TT	1107-1129			
CPV2_2F	TCC ACA TGA CAA AAG AAA GTG G	928-949	683	50	This study
CPV2_2R	ACC AGC TTC TTC AAT CCA AA	1591-1610			
CPV2_3F	GCA TGT GTT TTA AAT AGA CAA GGT G	1416-1440	695	52	This study
CPV2_3R	GTA CTC CAC GGT TCC AGT GC	2091-2110			
CPV2_4F	CAT CAT TGG GGA AAA GTR CCA	1926-1946	710	48	This study
CPV2_4R	GGA GCA ATT GCC TTT TTA GC	2636-2617			
CPV2_5F	AAT ATC TTG GGC CTG GGA AC	2408-2427	725	50	This study
CPV2_5R	AGC ATT TGC ATC AAC CAA TG	3113-3132			
CPV2_6F	AAT TTT TGG AAA ACG GAT GG	2939-2958	691	48	This study
CPV2_6R	TTT GTT TGC CAT GTR TGT GTT	3629-3623			
CPV2_7F	TGG AGA TAT TAT TTT CAA TGG GAT A	3406-3430	706	50	This study
CPV2_7R	TAA TTC CTG YTT TAC CTC CAA	4091-4111			
CPV2_8F	GGT AGA CAA CAT GGT CAA AAA AC	3925- 3947	833	50	This study
CPV2_8R	ACC ACC CAC ACC ATA ACA AC	4738-4757			

\* PCR-RFLP primers for identification of CPV-2c with product size 583 bp, CPV-2c can be digested with *MbolI* into 500 bp and 83 bp.

\*\* primers for identification of CPV-2a and CPV-2b with product size 681 bp

\*\*\*primers for identification of CPV-2b with product size 427 bp

## 7.4. Results

From September 2016 to April 2018, a viral enteric disease surveillance in domestic dogs and cats was conducted in four provinces of Thailand. Of 444 canine samples and 63 feline samples subjected to parvovirus identification, the positivity of CPV-2 in dogs was 29.95% (133/444) and FPV in cats was 58.73% (37/63), which were high in non-vaccinated animals (44.59%). Moreover, animals of young age (<1 year) were more frequently infected with CPV-2 (45.96%) (Table 7.2 and Table 7.3). In this study, all samples were also examined for other important enteric viruses including Canine Rotavirus (CRV) and Canine Coronavirus (CoV). We found co-infection of CPV-2 and CRV (n=1) as well as CPV-2 and CoV (n=22) in dogs. Additionally, coinfection of FPV and CoV was observed in two cats (data not shown).

**Table 7.2.** Association between age and clinical presentations of CPV-2 and FPV detection in this study

Age	Dogs		Cats	
	CPV-2 positive (%)	FPV positive (%)	Asymptomatic	Clinical sign
Young (< 1 year)	0/12 (0%)	91/198 (45.96%)	2/3 (66.67%)	28/47 (59.57%)
Adult (1-5 years)	3/63 (4.76%)	23/104 (22.12%)	0/0 (0%)	6/11 (54.55%)
Older (>5 years)	0/3 (0%)	16/64 (25.00%)	0/0 (0%)	1/2 (50.00%)
	<b>3/78 (3.84%)</b>	<b>130/366 (35.52%)</b>	<b>2/3 (66.67%)</b>	<b>35/60 (58.33%)</b>

**Table 7.3.** Association between vaccine history and clinical presentations of CPV-2 and FPV detection in this study

Vaccine history	Dogs		Cats	
	CPV-2 positive (%)		FPV positive (%)	
	Asymptomatic	Clinical sign	Asymptomatic	Clinical sign
Non-vaccination	0/67 (0%)	103/231 (44.59%)	2/3 (66.67%)	34/53 (64.15%)
Completed	3/11 (27.27%)	27/135 (20.00%)	0/0 (0%)	1/7 (14.29%)
	<b>3/78 (3.85%)</b>	<b>130/366 (35.52%)</b>	<b>2/3 (66.67%)</b>	<b>35/60 (58.33%)</b>

In this study, we identified antigenic types of CPV-2 as CPV-2c (n = 62; 46.61%), CPV-2a (n = 68; 51.13%) and CPV-2b (n = 3; 2.26%) (Table 7.4). It is noted that both CPV-2c and CPV-2a were predominant variants and CPV-2c has never been reported in Thailand. In this study, we selected 34 parvoviruses for genetic characterization. For CPV-2, the viruses were subjected to VP2 gene (n = 21) and whole genome sequencing (n = 2; Dog/ CU-24 and Cat/CU-21). For FPV, the viruses were subjected to VP2 gene sequencing (n = 11). The nucleotide sequences of the parvoviruses were submitted to the GenBank database under accession no. MH711880–MH711913 (Table 7.5). Pairwise comparisons of nucleotide and deduced amino acid sequences of Thai viruses were performed against those of vaccine and reference strains. Our results showed that the whole genomes of two Thai-CPV-2 (Dog/CU-24 and Cat/CU-21) had 99.90% nucleotide identity to each other and the highest nucleotide identities to Vietnam CPV-2c (99.60% at WG, 99.90% at VP2) (Table 7.6). Within Thai-CPV-2, the VP2 gene is diverse with nucleotide identities of 99.80%–100% (CPV-2c), 99.00%–99.20% (CPV-2b-297A) and 98.80%–99.00% (CPV-2a-297A) (Table 7.7). In this study, the overall dN/dS ratio for the partial VP2 of CPV-2

and FPV was lower than 1 (0.296, 0.032), implying that the gene was under negative selection or purifying selection as the main evolutionary force.





**Table 7.4.** Detail of sample tested and CPV2 variants in this study.

Year	Month	CPV2 positive (%)	CPV-2a (%)	CPV-2b (%)	CPV-2c (%)
2016	Sep	3/17 (17.65)	1 (33.33)*	0	2 (66.67)
	Oct	9/44 (20.45)	8 (89.89)	0	1 (11.11)
	Nov	6/19 (31.58)	3 (50.00)	0	3 (50.00)
	Dec	4/13 (30.77)	2 (50.00)	1 (25.00)	1 (25.00)
2017	Jan	5/44 (11.36)	2 (40.00)	0	3 (60.00)
	Feb	7/55 (12.73)	5 (71.43)	0	2 (28.57)
	Mar	0/10 (0)	0	0	0
	Apr	4/7 (57.14)	2 (50.00)	0	2 (50.00)
	May	1/3 (33.33)	1 (100.00)	0	0
	Jun	10/10 (100.00)	0	0	10 (100.00)
	Jul	10/12 (83.33)	2 (20.00)	0	8 (80.00)
	Aug	4/4 (100.00)	2 (50.00)	0	2 (50.00)
	Sep	10/22 (45.45)	8 (80.00)	1 (10.00)	1 (10.00)
	Oct	0/11 (0)	0	0	0
	Nov	2/19 (10.53)	1 (50.00)	1 (50.00)	0
	Dec	5/33 (15.15)	2 (40.00)	0	3 (60.00)
2018	Jan	9/30 (30.00)	4 (44.44)	0	5 (55.56)
	Feb	23/36 (63.89)	14 (60.87)	0	9 (39.13)
	Mar	10/25 (40)	5 (50.00)	0	5 (50.00)
	Apr	11/30 (36.67)	6 (54.55)	0	5 (45.45)
		<b>133*/444 (29.95)</b>	<b>68 (51.13)</b>	<b>3 (2.26)</b>	<b>62 (46.61)</b>

\* one isolate is identified as FPV

**Table 7.5.** Detailed descriptions of CPV-2 and FPV characterized in this study.

Virus	Breed	Age	Vaccine History	Clinical Sign	Collection Date	Location	Type of CPV/FPV	GenBank #
<b>CPV</b>								
Dog/Thailand/CU-41/2016	Mixed	2 years	C	Asymptomatic	Oct-16	Bangkok	CPV-2a-297A	MH711880
Dog/Thailand/CU-53/2016	Pomeranian	2 month	I	Diarrhea	Oct-16	Bangkok	CPV-2a-297A	MH711881
Dog/Thailand/CU-54/2016	Yorkshire terria	1 years	C	Diarrhea	Oct-16	Bangkok	CPV-2a-297A	MH711882
Dog/Thailand/CU-57/2016	Pomeranian	2 month	I	Diarrhea	Oct-16	Bangkok	CPV-2a-297A	MH711883
Dog/Thailand/CU-60/2016	Pomeranian	2 month	I	Diarrhea	Oct-16	Bangkok	CPV-2a-297A	MH711884
Dog/Thailand/CU-70/2016	Siberian husky	4 month	I	Diarrhea	Oct-16	Bangkok	CPV-2a-297A	MH711885
Dog/Thailand/CU-245/2017	Mixed	2 month	I	Diarrhea	Apr-17	Bangkok	CPV-2a-297A	MH711886
Dog/Thailand/CU-246/2017	Beagle	2 month	I	Diarrhea	Apr-17	Bangkok	CPV-2a-297A	MH711887
Dog/Thailand/CU-281/2017	Mixed	3 month	I	Diarrhea	Sep-17	Bangkok	CPV-2a-297A	MH711888
Dog/Thailand/CU-287/2017	Mixed	1 years	I	Diarrhea	Sep-17	Bangkok	CPV-2a-297A	MH711889
Dog/Thailand/CU-101/2016	Pekingese	2 month	I	Diarrhea	Dec-16	Bangkok	CPV-2b-297A	MH711890
Dog/Thailand/CU-20139/2017	Beagle	2 month	I	Diarrhea	Nov-17	Bangkok	CPV-2b-297A	MH711891
Dog/Thailand/CU-10/2016	Beagle	2 years	C	Asymptomatic	Sep-16	Bangkok	CPV-2c	MH711892
Dog/Thailand/CU-16/2016	Shih Tzu	2 month	I	Diarrhea	Sep-16	Bangkok	CPV-2c	MH711893
Dog/Thailand/CU-24/2016	Mixed	2 years	C	Asymptomatic	Oct-16	Bangkok	CPV-2c	MH711894*
Dog/Thailand/CU-81/2016	Chihuahua	2 month	I	Diarrhea	Nov-16	Bangkok	CPV-2c	MH711895
Dog/Thailand/CU-155/2017	Pomeranian	6 month	I	Diarrhea	Jan-17	Bangkok	CPV-2c	MH711896
Dog/Thailand/CU-247/2017	Jack russian	2 month	I	Diarrhea	Apr-17	Bangkok	CPV-2c	MH711897
Dog/Thailand/CU-255/2017	German Shepherd	2 month	I	Diarrhea	Jun-17	N.Ratchasima	CPV-2c	MH711898
Dog/Thailand/CU-256/2017	German Shepherd	2 month	I	Diarrhea	Jun-17	N.Ratchasima	CPV-2c	MH711899
Dog/Thailand/CU-257/2017	German Shepherd	2 month	I	Diarrhea	Jun-17	N.Ratchasima	CPV-2c	MH711900
Dog/Thailand/CU-267/2017	Mixed	4 month	I	Diarrhea	Jul-17	Tak	CPV-2c	MH711901
Cat/Thailand/CU-21/2016	Mixed	5 month	I	Diarrhea	Oct-16	Bangkok	CPV-2c	MH711902*
<b>FPV</b>								
Cat/Thailand/CU-80/2016	Mixed	6 month	I	Diarrhea	Nov-16	Bangkok	FPV-G2	MH711903
Cat/Thailand/CU-18/2016	Mixed	5 month	I	Diarrhea	Sep-16	Bangkok	FPV-G1	MH711904
Cat/Thailand/CU-20/2016	Mixed	5 month	I	Diarrhea	Sep-16	Bangkok	FPV-G1	MH711905
Cat/Thailand/CU-98/2016	Mixed	2 month	I	Diarrhea	Dec-16	Bangkok	FPV-G1	MH711906
Cat/Thailand/CU-123/2017	Mixed	9 month	I	Asymptomatic	Jan-17	Chiang mai	FPV-G1	MH711907
Cat/Thailand/CU-154/2017	Mixed	3 month	I	Diarrhea	Jan-17	Bangkok	FPV-G1	MH711908
Cat/Thailand/CU-196/2017	Mixed	1 years	I	Diarrhea	Feb-17	Bangkok	FPV-G1	MH711909
Cat/Thailand/CU-220/2017	Mixed	3 month	I	Diarrhea	Feb-17	Bangkok	FPV-G1	MH711910
Cat/Thailand/CU-20143/2017	Mixed	2 month	I	Diarrhea	Nov-17	Bangkok	FPV-G1	MH711911
Cat/Thailand/CU-20246/2018	Mixed	5 month	I	Diarrhea	Jan-18	Bangkok	FPV-G1	MH711912
Dog/Thailand/CU-17/2016	Labrador retriever	13 years	C	Diarrhea	Sep-16	Bangkok	FPV-G1	MH711913

\* Whole genome sequence

**Table 7.6.** Pairwise comparisons of whole genome of Dog/Thailand/CU-24 and Cat/Thailand/CU-21 with those of reference CPV and FPV

Strain	Type	Accessions number	Year	Country	Dog/Thailand/CU-24/2016				
					% Nucleotide identity (% Amino acid identity)				
					WGS (4269 nt)	NS1 (2700 nt)	NS2 (498 nt)	VP1 (2184 nt)	VP2 (1755 nt)
Dog/Thailand/CU-24/2016	CPV-2c	This study	2016	Thailand	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)
Cat/Thailand/CU-21/2016	CPV-2c	This study	2016	Thailand	99.90 (99.40)	100.00 (99.70)	99.80 (98.80)	100.00 (99.70)	100.00 (99.80)
<b>Reference CPV</b>									
CPV-2/Dog/USA/CPV-5/1979	CPV-2	EU659116	1979	USA	99.10 (98.50)	99.30 (99.10)	98.60 (97.60)	99.20 (98.60)	98.90 (97.90)
CPV-2/Dog/USA/CPV-6/1980	CPV-2	EU659117	1980	USA	99.10 (98.50)	99.30 (99.10)	98.60 (97.60)	99.20 (98.60)	98.90 (97.90)
CPV-2/Dog/xxx/CPV-N/1995	CPV-2	M19296	1995	N/A	99.10 (98.50)	99.30 (99.10)	98.60 (98.20)	99.10 (98.60)	98.90 (97.90)
CPV/Vaccine	CPV-2	FJ197846	2007	South Korea	N/A	N/A	N/A	N/A	98.70 (97.60)
CPV-2a/Dog/USA/CPV-13/1981	CPV-2a	EU659118	1981	USA	99.20 (98.90)	99.30 (99.10)	98.60 (97.60)	99.20 (99.00)	99.00 (98.80)
CPV-2a/Dog/Japan/Y1/xxxx	CPV-2a	D26079	N/A	Japan	99.20 (98.90)	99.20 (99.10)	98.60 (97.60)	99.20 (99.00)	99.10 (98.80)
CPV-2a/Dog/Thailand/KU14/2008	CPV-2a-297A	GQ379043	2008	Thailand	N/A	N/A	N/A	N/A	99.20 (99.10)
CPV-2a /Dog/China/SC02/2011	CPV-2a-297A	JX660690	2011	China	99.10 (98.70)	99.10 (98.80)	98.60 (97.60)	99.20 (98.90)	99.10 (99.00)
CPV-2b/Dog/Italy/1-99/1999	CPV-2b	MF177226	1999	Italy	99.20 (98.80)	99.20 (99.00)	98.80 (98.20)	99.30 (98.90)	99.20 (98.80)
CPV-2b/Dog/USA/CPV-411b/1998	CPV-2b-297A	EU659121	1998	USA	99.10 (99.00)	99.10 (99.10)	98.60 (97.60)	99.10 (99.10)	99.00 (99.00)
CPV/Vaccine	CPV-2b	FJ222822	N/A	N/A	N/A	N/A	N/A	N/A	99.30 (98.60)
CPV-2c/Dog/USA/OH20219/2015	CPV-2c	MF457594	2015	USA	99.00 (98.60)	99.00 (98.70)	98.60 (98.20)	99.00 (98.70)	99.00 (99.00)
CPV-2c/Dog/ Italy/288-01/2001	CPV-2c	MF177239	2001	Italy	99.30 (99.10)	99.20 (99.10)	98.80 (99.10)	99.30 (99.10)	99.30 (99.10)
CPV-2c/Dog/ Viet Nam/HCM/7/2013	CPV-2c	LC214969	2013	Viet Nam	99.60 (99.20)	99.70 (99.40)	99.60 (98.80)	99.70 (99.50)	99.90 (99.80)
CPV-2c/Dog/ Indonesia/HCM/14/2013	CPV-2c	LC216909	2013	Indonesia	N/A	N/A	N/A	N/A	99.80 (99.80)
<b>Reference FPV</b>									
FPV/Cat/USA-4/1964	FPV	EU659112	1964	USA	98.40 (97.70)	98.60 (98.40)	98.00 (95.80)	98.60 (98.00)	98.20 (97.10)
FPV/Cat/USA/kai/2006	FPV	EU659115	2006	USA	98.50 (97.80)	98.80 (98.70)	98.20 (96.40)	98.50 (97.90)	98.20 (97.10)
FPV/Cat/Italy/42/06-G2/2006	FPV	EU498698	2006	Italy	N/A	N/A	N/A	N/A	98.00 (96.90)
FPV/Vaccine	FPV	EU498681	N/A	N/A	N/A	N/A	N/A	N/A	98.20 (97.10)

\* N/A = not available

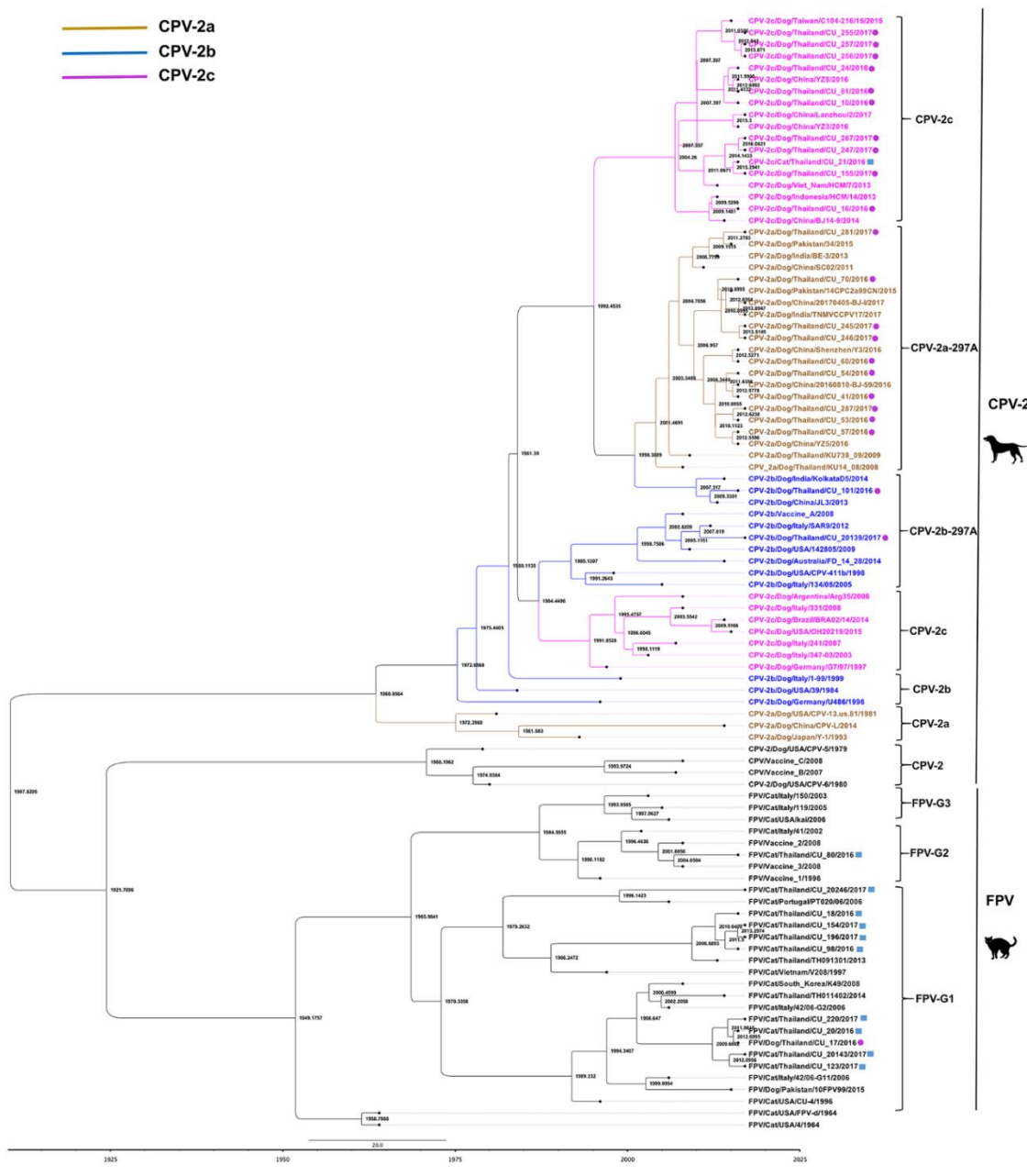
**Table 7.7.** Pairwise comparisons of partial VP2 gene of Dog/Thailand/CU-24 with Thai-CPV and FPV and reference CPV and FPV.

Strain	Type	Accession Number	Year	Country	Partial VP2 gene of Dog/Thailand/CU-24/2016	
					%Nucleotide identity (551nt)	% Amino acid identity (167 aa)
<b>This study CPV</b>						
CPV-2c/Dog/Thailand/CU 24/2016	CPV-2c		2016	Thailand	100.00	100.00
CPV-2a/Dog/Thailand/CU 41/2016	CPV-2a-297A		2016	Thailand	99.00	98.80
CPV-2a/Dog/Thailand/CU 53/2016	CPV-2a-297A		2016	Thailand	99.00	98.80
CPV-2a/Dog/Thailand/CU 54/2016	CPV-2a-297A		2016	Thailand	99.00	98.80
CPV-2a/Dog/Thailand/CU 57/2016	CPV-2a-297A		2016	Thailand	99.00	98.80
CPV-2a/Dog/Thailand/CU 60/2016	CPV-2a-297A		2016	Thailand	99.00	98.80
CPV-2a/Dog/Thailand/CU 70/2016	CPV-2a-297A		2016	Thailand	99.00	98.80
CPV-2a/Dog/Thailand/CU 245/2017	CPV-2a-297A		2017	Thailand	98.80	98.80
CPV-2a/Dog/Thailand/CU 246/2017	CPV-2a-297A		2017	Thailand	98.80	98.80
CPV-2a/Dog/Thailand/CU 281/2017	CPV-2a-297A		2017	Thailand	98.80	98.80
CPV-2a/Dog/Thailand/CU 287/2017	CPV-2a-297A		2017	Thailand	99.00	98.80
CPV-2b/Dog/Thailand/CU 101/2016	CPV-2b-297A		2016	Thailand	99.20	98.80
CPV-2b/Dog/Thailand/CU 20139/2017	CPV-2b-297A		2017	Thailand	99.00	97.60
CPV-2c/Dog/Thailand/CU 10/2016	CPV-2c		2016	Thailand	100.00	100.00
CPV-2c/Dog/Thailand/CU 16/2016	CPV-2c		2016	Thailand	100.00	100.00
CPV-2c/Cat/Thailand/CU 21/2016	CPV-2c		2016	Thailand	100.00	100.00
CPV-2c/Dog/Thailand/CU 81/2016	CPV-2c		2016	Thailand	100.00	100.00
CPV-2c/Dog/Thailand/CU 155/2017	CPV-2c		2017	Thailand	100.00	100.00
CPV-2c/Dog/Thailand/CU 247/2017	CPV-2c		2017	Thailand	100.00	100.00
CPV-2c/Dog/Thailand/CU 255/2017	CPV-2c		2017	Thailand	99.80	100.00
CPV-2c/Dog/Thailand/CU 256/2017	CPV-2c		2017	Thailand	99.80	100.00
CPV-2c/Dog/Thailand/CU 257/2017	CPV-2c		2017	Thailand	99.80	100.00
CPV-2c/Dog/Thailand/CU 267/2017	CPV-2c		2017	Thailand	100.00	100.00
<b>Reference CPV</b>						
CPV-2/Dog/USA/CPV-5/1979	CPV-2	EU659116	1979	USA	98.00	95.80
CPV-2/Dog/USA/CPV-6/1980	CPV-2	EU659117	1980	USA	98.00	95.80
CPV-2/Dog/xxx/CPV-N/1995	CPV-2	M19296	1995	N/A	97.80	95.80
CPV/Vaccine	Vaccine	FJ197846	2007	South Korea	97.80	95.20
CPV-2a/Dog/USA/CPV-13/1981	CPV-2a	EU659118	1981	USA	98.60	97.60
CPV-2a/Dog/Japan/Y1/xxxx	CPV-2a	D26079	N/A	Japan	98.40	97.60
CPV-2a/Dog/Thailand/KU14/2008	CPV-2a-297A	GQ379043	2008	Thailand	98.80	98.80
CPV-2a /Dog/China/SC02/2011	CPV-2a-297A	JX660690	2011	China	99.00	98.80
CPV-2b/Dog/Italy/1-99/1999	CPV-2b	MF177226	1999	Italy	98.80	97.60
CPV-2b/Dog/USA/CPV-411b/1998	CPV-2b-297A	EU659121	1998	USA	98.80	98.20
CPV-2b/Dog/India/KolkataD5/2014	CPV-2b-297A	KP071953	2014	India	99.20	98.80
CPV/Vaccine	Vaccine	FJ222822	N/A	N/A	99.00	97.60

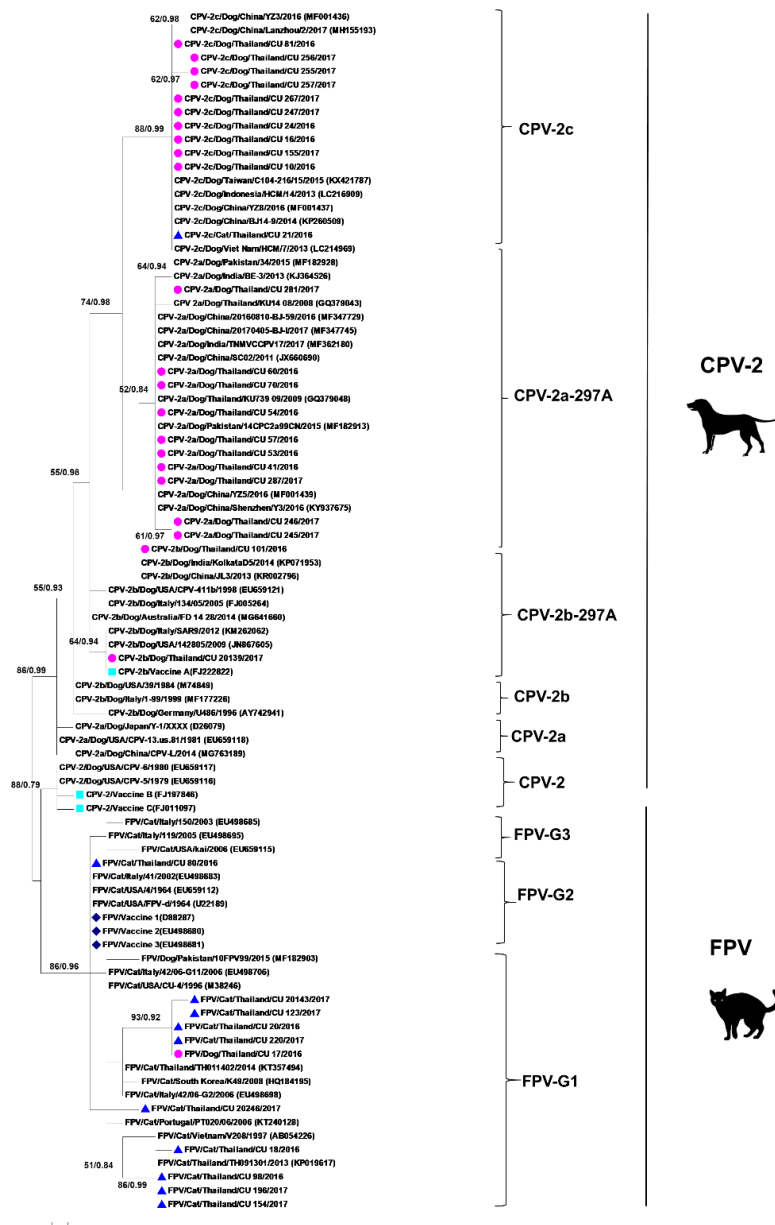
**Table 7.7.** Pairwise comparisons of partial VP2 gene of Dog/Thailand/CU-24 with Thai-CPV and FPV and reference CPV and FPV. (cont.)

Strain	Type	Accession Number	Year	Country	Partial VP2 gene of Dog/Thailand/CU-24/2016	
					%Nucleotide identity (551nt)	% Amino acid identity (167 aa)
<b>Reference CPV</b>						
CPV/Vaccine	Vaccine	FJ197846	2007	South Korea	97.80	95.20
CPV-2a/Dog/USA/CPV-13/1981	CPV-2a	EU659118	1981	USA	98.60	97.60
CPV-2a/Dog/Japan/Y1/xxxx	CPV-2a	D26079	N/A	Japan	98.40	97.60
CPV-2a/Dog/Thailand/KU14/2008	CPV-2a-297A	GQ379043	2008	Thailand	98.80	98.80
CPV-2a /Dog/China/SC02/2011	CPV-2a-297A	JX660690	2011	China	99.00	98.80
CPV-2b/Dog/Italy/1-99/1999	CPV-2b	MF177226	1999	Italy	98.80	97.60
CPV-2b/Dog/USA/CPV-411b/1998	CPV-2b-297A	EU659121	1998	USA	98.80	98.20
CPV-2b/Dog/India/KolkataD5/2014	CPV-2b-297A	KP071953	2014	India	99.20	98.80
CPV/Vaccine	Vaccine	FJ222822	N/A	N/A	99.00	97.60
CPV-2c/Dog/USA/OH20219/2015	CPV-2c	MF457594	2015	USA	99.20	98.80
CPV-2c/Dog/ Italy/288-01/2001	CPV-2c	MF177239	2001	Italy	99.20	98.80
CPV-2c/Dog/ Viet Nam/HCM/7/2013	CPV-2c	LC214969	2013	Viet Nam	100.00	100.00
CPV-2c/Dog/ Indonesia/HCM/14/2013	CPV-2c	LC216909	2013	Indonesia	100.00	100.00
CPV-2c/Dog/Taiwan/C104-216/2015	CPV-2c	KX421787	2015	Taiwan	100.00	100.00
<b>This study FPV</b>						
FPV/Cat/Thailand/CU 18/2016	FPV-G1		2016	Thailand	96.60	95.80
FPV/Cat/Thailand/CU 20/2016	FPV-G1		2016	Thailand	97.00	95.80
FPV/Cat/Thailand/CU 80/2016	FPV-G2		2016	Thailand	97.60	95.80
FPV/Cat/Thailand/CU 98/2016	FPV-G1		2016	Thailand	96.80	95.80
FPV/Cat/Thailand/CU 123/2017	FPV-G1		2017	Thailand	96.80	95.80
FPV/Cat/Thailand/CU 154/2017	FPV-G1		2017	Thailand	96.80	95.80
FPV/Cat/Thailand/CU 196/2017	FPV-G1		2017	Thailand	96.80	95.80
FPV/Cat/Thailand/CU 220/2017	FPV-G1		2017	Thailand	97.00	95.80
FPV/Cat/Thailand/CU 20143/2017	FPV-G1		2017	Thailand	96.80	95.80
FPV/Cat/Thailand/CU 20246/2017	FPV-G1		2017	Thailand	97.00	95.80
FPV/Dog/Thailand/CU 17/2016	FPV-G1		2016	Thailand	97.00	95.80
<b>Reference FPV</b>						
FPV/Cat/USA-4/1964	FPV	EU659112	1964	USA	97.60	95.80
FPV/Cat/USA/kai/2006	FPV	EU659115	2006	USA	97.40	95.80
FPV/Cat/Italy/42/06-G2/2006	FPV	EU498698	2006	Italy	97.20	95.80
FPV/Vaccine	FPV	EU498681	N/A	N/A	97.60	95.80

Phylogenetic analysis of the VP2 gene from Thai-CPV-2 showed that the viruses were clustered with CPV-2c, CPV-2a-297A and CPV-2b-297A. The phylogenetic analysis indicated that Thai-CPV-2c was closely related to VietNam-HCM7, Chinese-YZ-8, BJ14-9, Taiwan-C104 and Indonesia-HCM but was in separate subclusters from the North American and European CPV-2c (Figure 7.1 and Figure 7.2). Based on the MCC tree, the Asian-CPV-2c was estimated to separate from CPV-2C of America and Europe since 1981. While Thai-CPV-2c was started to evolve from other Asian-CPV-2c viruses (China, Taiwan, Vietnam, and Indonesia) since 2004. The estimated nucleotide substitution rate of the partial VP2 was  $1.1905 \times 10^{-4}$  substitutions per site per year. 95% highest posterior densities (HPD) were  $6.9511 \times 10^{-5}$ – $1.6877 \times 10^{-4}$ . It is noted that the new variant CPV-2b-297A (n = 2) was clustered in a separate group in which one isolate (Dog/CU-20139) was closely related to the vaccine strain (CPV-2b/Vaccine), suggesting a virus of vaccine origin. The phylogenetic analysis of the VP2 gene of FPV was also performed, showing that Thai-FPV was predominantly clustered with FPV-G1 (n=10), including one canine isolate (Dog/CU-17). In contrast, one Thai-FPV (Dog/CU-80) was grouped in a distinct cluster (G2) with FPV vaccine strains (Figure 7.2). It is interesting to note that one dog isolate was clustered with FPV-G1, suggesting FPV infection in a dog.



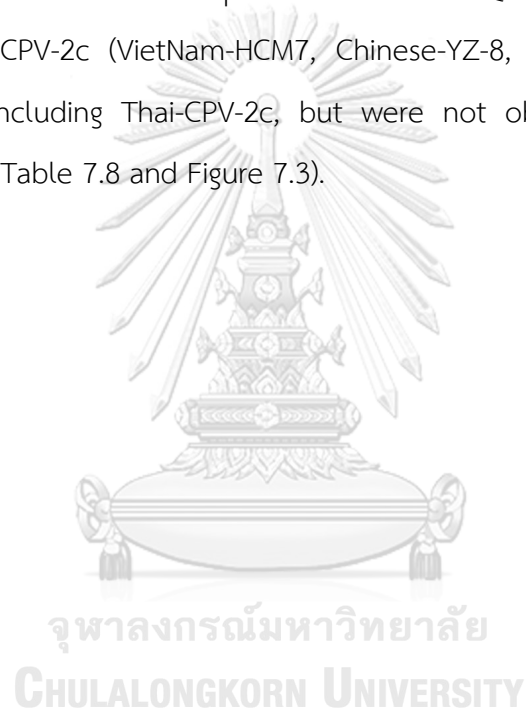
**Figure 7.1.** Phylogenetic tree of VP2 gene of canine parvovirus type 2 and feline parvovirus. Circles and squares represent Thai-CPV-2 and FPV, respectively. The phylogenetic tree was constructed by using the Beast program with Bayesian Markov-Chain Monte Carlo (BMCMC), with 10,000,000 generations and an average standard deviation of split frequencies



**Figure 7.2.** Phylogenetic tree of VP2 gene sequences of the canine parvovirus type 2 (CPV-2a, CPV-2b, CPV-2c, CPV-2a-297A, CPV-2b-297A) and feline parvovirus. Thai-CPV-2 and FPV (n=34) are included in the phylogenetic tree. Circles and triangles represent Thai- CPV-2 and FPV, respectively. Squares represent CPV and FPV vaccine strains. Phylogenetic tree was constructed by using MEGA v.6.06 program with neighbor-joining algorithm applying bootstrap analysis with 1,000 replications.



Genetic analyses of the genomes of Thai-CPV-2 and FPV were also conducted (Table 7.8). CPV-2a, CPV-2b and CPV-2c variants were determined by genetic differences at VP2 position 426 as Asn (N), Asp (D) and Glu (E), respectively (Martella et al., 2006). In this study, the new variants CPV-2a-297A and CPV-2b-297A, had unique amino acids at positions 297A, 426N and 426D, which were also observed in reference viruses. Similarly, Thai- CPV-2c contained unique amino acids at positions 297A and 426E, which were observed in reference CPV-2c. It is important to note that unique amino acid substitutions at positions Y324I and Q370R were only observed in the Asian strain CPV-2c (VietNam-HCM7, Chinese-YZ-8, BJ14-9, Taiwan-C104 and Indonesia-HCM), including Thai-CPV-2c, but were not observed in American and European CPV2-c (Table 7.8 and Figure 7.3).



**Table 7.8.** Genetic analysis of deduced amino acids of Thai-CPV-2 and FPV in comparison to those of vaccine and reference strains

Strain	Accession number	Year	Country	Amino acid position of VP2 gene										Type
				Typing				Important amino acids						
				297	426	300 <sup>a</sup>	305 <sup>a</sup>	321	323 <sup>b</sup>	324	370 <sup>c</sup>	371	375	
<b>Reference CPV</b>														
CPV-2/Dog/USA/CPV-5/1979	EU659116	1979	USA	S	N	A	D	N	N	Y	Q	A	N	CPV-2
CPV-2/Dog/USA/CPV-6/1980	EU659117	1980	USA	S	N	A	D	N	N	Y	Q	A	N	CPV-2
CPV-2/Vaccine B (Nobivac; Intervet)	FJ197846	2007	South Korea	S	N	A	D	N	N	Y	Q	A	N	CPV-2/Vaccine
CPV-2/Vaccine C (Vaccine06; Merial)	FJ222822	N/A	N/A	A	D	G	Y	K	N	Y	Q	A	D	CPV-2/Vaccine
CPV-2a/Dog/USA/CPV-13/1981	EU659118	1981	USA	S	N	G	Y	N	N	Y	Q	A	D	CPV-2a
CPV-2a/Dog/Japan/Y1/xxxx	D26079	N/A	Japan	S	N	G	Y	N	N	Y	Q	A	D	CPV-2a
CPV-2a/Dog/Thailand/KU14/2008	GQ379043	2008	Thailand	A	N	G	Y	N	N	I	Q	A	D	CPV-2a-297A
CPV-2a /Dog/China/SC02/2011	JX660690	2011	China	A	N	G	Y	N	N	I	Q	A	D	CPV-2a-297A
CPV-2b/Dog/Italy/1-99/1999	MF177226	1999	Italy	S	D	G	Y	N	N	Y	Q	A	D	CPV-2b
CPV-2b/Dog/USA/CPV-411b/1998	EU659121	1998	USA	A	D	G	Y	N	N	Y	Q	A	D	CPV-2b-297A
CPV-2b/Dog/India/KolkataD5/2014	KP071953	2014	India	A	D	G	Y	N	N	I	Q	A	D	CPV-2b-297A
CPV-2b/Vaccine A (Duramune; Fort Dodge)	FJ222822	N/A	N/A	A	D	G	Y	K	N	Y	Q	A	D	CPV-2b/Vaccine
CPV-2c/Dog/Italy/288-01/2001	MF177239	2001	Italy	A	E	G	Y	N	N	Y	Q	A	D	CPV-2c
CPV-2c/Dog/USA/OH20219/2015	MF457594	2015	USA	A	E	G	Y	N	N	Y	Q	A	D	CPV-2c
CPV-2c/Dog/ VietNam/HCM/7/2013	LC214969	2013	Viet Nam	A	E	G	Y	N	N	I	R	A	D	CPV-2c
CPV-2c/Dog/Indonesia/HCM/14/2013	LC216909	2013	Indonesia	A	E	G	Y	N	N	I	R	A	D	CPV-2c
CPV-2c/Dog/Taiwan/C104-216/2015	KX421787	2015	Taiwan	A	E	G	Y	N	N	I	R	A	D	CPV-2c
<b>This study: CPV</b>														
CPV-2a/Dog/Thailand/CU 41/2016	This study	2016	Thailand	A	N	G	Y	N	N	I	Q	A	D	CPV-2a-297A
CPV-2a/Dog/Thailand/CU 53/2016	This study	2016	Thailand	A	N	G	Y	N	N	I	Q	A	D	CPV-2a-297A
CPV-2a/Dog/Thailand/CU 54/2016	This study	2016	Thailand	A	N	G	Y	N	N	I	Q	A	D	CPV-2a-297A
CPV-2a/Dog/Thailand/CU 57/2016	This study	2016	Thailand	A	N	G	Y	N	N	I	Q	A	D	CPV-2a-297A
CPV-2a/Dog/Thailand/CU 60/2016	This study	2016	Thailand	A	N	G	Y	N	N	I	Q	A	D	CPV-2a-297A
CPV-2a/Dog/Thailand/CU 70/2016	This study	2016	Thailand	A	N	G	Y	N	N	I	Q	A	D	CPV-2a-297A
CPV-2a/Dog/Thailand/CU 245/2017	This study	2017	Thailand	A	N	G	Y	N	N	I	Q	A	D	CPV-2a-297A
CPV-2a/Dog/Thailand/CU 246/2017	This study	2017	Thailand	A	N	G	Y	N	N	I	Q	A	D	CPV-2a-297A
CPV-2a/Dog/Thailand/CU 281/2017	This study	2017	Thailand	A	N	G	Y	N	N	I	Q	A	D	CPV-2a-297A
CPV-2a/Dog/Thailand/CU 287/2017	This study	2017	Thailand	A	N	G	Y	N	N	I	Q	A	D	CPV-2a-297A
CPV-2b/Dog/Thailand/CU 101/2016	This study	2016	Thailand	A	D	G	Y	N	N	I	Q	A	D	CPV-2b-297A
CPV-2b/Dog/Thailand/CU 20139/2017	This study	2017	Thailand	A	D	G	Y	K	N	Y	Q	A	D	CPV-2b-297A
CPV-2c/Dog/Thailand/CU 10/2016	This study	2016	Thailand	A	E	G	Y	N	N	I	R	A	D	CPV-2c
CPV-2c/Dog/Thailand/CU 16/2016	This study	2016	Thailand	A	E	G	Y	N	N	I	R	A	D	CPV-2c
CPV-2c/Dog/Thailand/CU 24/2016	This study	2016	Thailand	A	E	G	Y	N	N	I	R	A	D	CPV-2c
CPV-2c/Dog/Thailand/CU 81/2016	This study	2016	Thailand	A	E	G	Y	N	N	I	R	A	D	CPV-2c
CPV-2c/Dog/Thailand/CU 155/2017	This study	2017	Thailand	A	E	G	Y	N	N	I	R	A	D	CPV-2c
CPV-2c/Dog/Thailand/CU 247/2017	This study	2017	Thailand	A	E	G	Y	N	N	I	R	A	D	CPV-2c
CPV-2c/Dog/Thailand/CU 255/2017	This study	2017	Thailand	A	E	G	Y	N	N	I	R	A	D	CPV-2c
CPV-2c/Dog/Thailand/CU 256/2017	This study	2017	Thailand	A	E	G	Y	N	N	I	R	A	D	CPV-2c
CPV-2c/Dog/Thailand/CU 257/2017	This study	2017	Thailand	A	E	G	Y	N	N	I	R	A	D	CPV-2c
CPV-2c/Dog/Thailand/CU 267/2017	This study	2017	Thailand	A	E	G	Y	N	N	I	R	A	D	CPV-2c
CPV-2c/Cat/Thailand/CU 21/2016	This study	2016	Thailand	A	E	G	Y	N	N	I	R	A	D	CPV-2c <sup>a</sup>

**Table 7.8.** Genetic analysis of deduced amino acids of Thai-CPV-2 and FPV in comparison to those of vaccine and reference strains (cont.)

Strain	Accession number	Year	Country	Amino acid position of VP2 gene										Type
				Typing				Important amino acids						
				297	426	300 <sup>a</sup>	305 <sup>a</sup>	321	323 <sup>b</sup>	324	370 <sup>c</sup>	371	375	
<b>Reference FPV</b>														
FPV/Cat/USA-4/1964	EU659112	1964	USA	S	N	A	D	N	D	Y	Q	A	D	
FPV/Cat/USA/kai/2006	EU659115	2006	USA	S	N	A	D	N	D	Y	Q	A	D	
FPV/Cat/Italy/42/06-G2/2006	EU498698	2006	Italy	S	N	A	D	N	D	Y	Q	A	D	
FPV/Cat/Thailand/TH011402/2014	KT357494	2014	Thailand	S	N	A	D	N	D	Y	Q	A	D	
FPV/Dog/Pakistan/10FPV99/2015	MF182903	2015	Pakistan	S	N	A	D	N	D	Y	Q	A	D	
FPV/Vaccine 1 (PLH-V)	D88287	N/A	N/A	S	N	A	D	N	D	Y	Q	A	D	
FPV/Vaccine 2 (Purevax;Merial)	EU498680	N/A	N/A	S	N	A	D	N	D	Y	Q	A	D	
FPV/Vaccine 3 (Felocell;Pfizer)	EU498681	N/A	N/A	S	N	A	D	N	D	Y	Q	A	D	
<b>This study: FPV</b>														
FPV/Cat/Thailand/CU 80/2016	This study	2016	Thailand	S	N	A	D	N	D	Y	Q	A	D	FPV-G2
FPV/Cat/Thailand/CU 18/2016	This study	2016	Thailand	S	N	A	D	N	D	Y	Q	A	D	FPV-G1
FPV/Cat/Thailand/CU 20/2016	This study	2016	Thailand	S	N	A	D	N	D	Y	Q	A	D	FPV-G1
FPV/Cat/Thailand/CU 98/2016	This study	2016	Thailand	S	N	A	D	N	D	Y	Q	A	D	FPV-G1
FPV/Cat/Thailand/CU 123/2017	This study	2017	Thailand	S	N	A	D	N	D	Y	Q	A	D	FPV-G1
FPV/Cat/Thailand/CU 154/2017	This study	2017	Thailand	S	N	A	D	N	D	Y	Q	A	D	FPV-G1
FPV/Cat/Thailand/CU 196/2017	This study	2017	Thailand	S	N	A	D	N	D	Y	Q	A	D	FPV-G1
FPV/Cat/Thailand/CU 220/2017	This study	2017	Thailand	S	N	A	D	N	D	Y	Q	A	D	FPV-G1
FPV/Cat/Thailand/CU 20143/2017	This study	2017	Thailand	S	N	A	D	N	D	Y	Q	A	D	FPV-G1
FPV/Cat/Thailand/CU 20246/2017	This study	2017	Thailand	S	N	A	D	N	D	Y	Q	A	D	FPV-G1
FPV/Dog/Thailand/CU 17/2016	This study	2016	Thailand	S	N	A	D	N	D	Y	Q	A	D	FPV-G1 <sup>b</sup>

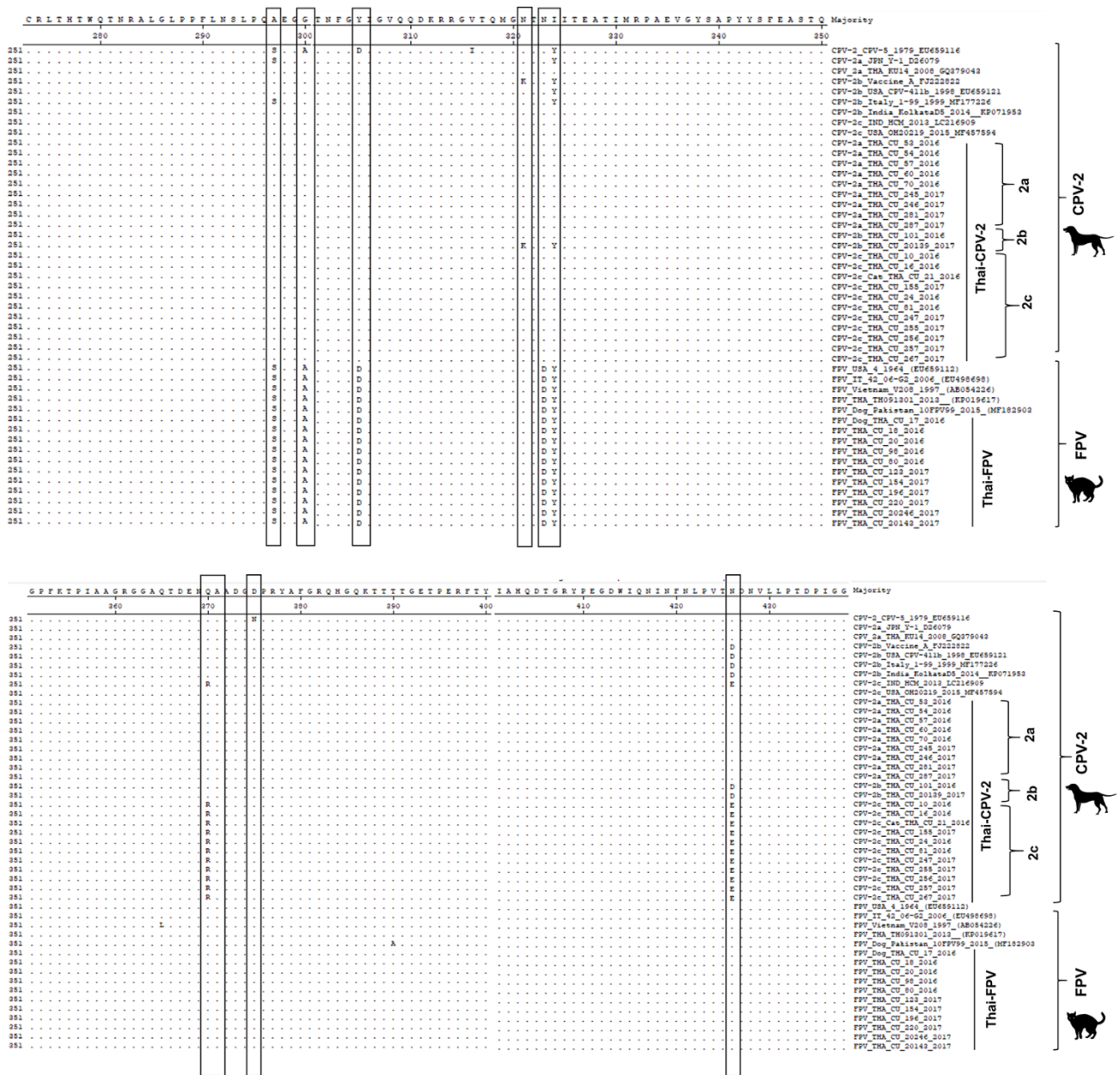


Figure 7.3. Amino acid alignment of VP2 protein of CPV-2. Dots represent matched amino acid residues. Open boxes indicate amino acid substitutions.

## 7.5. Discussion

To our knowledge, this study is the first to report CPV-2c in dogs and cats in Thailand. The infected animals showed clinical signs of acute hemorrhagic or watery diarrhea. In this study, the positivity of CPV-2 in dogs was 29.95% and that of FPV in cats was 58.73%, which were high in non-vaccinated animals. This study also showed that CPV-2 was predominantly detected in dogs of young age (<1 year). These results were similar to the previous report of CPV-2 in puppies in Thailand (Sakulwira et al., 2003). It is important to note that CPV-2c could also be isolated from cat. Similar observations were also reported in other studies (Miranda et al., 2014; Nakamura et al., 2001). One FPV infection in a dog was observed, as also seen in a previous study of FPV infection in sick dogs in Pakistan in 2018 (Ahmed et al., 2018).

Nucleotide and amino acid comparison showed that the whole genomes of two Thai-CPV-2 strains had 99.90% nucleotide identity to each other and had highest nucleotide identities to Asian-CPV-2c from Vietnam. Similar studies reported Asian-CPV-2c in China and Taiwan (Chiang et al., 2016; Guo et al., 2013). Phylogenetic analysis showed that Thai-CPV-2c is closely related to Asian CPV-2c including VietNam-HCM7, Chinese-YZ-8, BJ14-9, Taiwan-C104 and Indonesia-HCM. These viruses were in separated subclusters from North American and European CPV-2c. Our analysis suggested that the estimated time of the most recent common ancestor of Thai-CPV-2c subclusters was 2004 (Figure 7.1). The substitution rate of parvovirus in this study was in agreement with other studies ( $1.2 \times 10^{-4}$ – $2.2 \times 10^{-4}$  substitutions per site per year) (Hoelzer et al., 2008; Pereira et al., 2007; Shackelton et al., 2005). Moreover, our data indicated that parvovirus (which is DNA virus) has high genomic substitution rate similar to other RNA viruses at approximately  $10^{-4}$  substitutions per site per year (Duffy et al., 2008). Whole analysis indicated that Thai-CPVs are closely related to Asian-CPV-2c with unique amino acids at position 297A, 370R and 426E of VP2 suggesting predominant Asian-CPV-2c in the country. It is also noted that unique amino acid substitutions at position Y324I and Q370R were only observed in Asian strain CPV-2c. These unique amino acids (370R) might relate to receptor-binding

properties, suggesting species preference. Recent observations have also been reported in China and Taiwan (Chiang et al., 2016; Guo et al., 2013).

The identification of several types of CPV2 (CPV-2c, new variant CPV-2a-297A, and new variant CPV-2b-297A) demonstrates diversity of CPV2 in Thailand. CPV-2c is an emerging variant in the country and the Southeast Asia region. These findings will stimulate concern regarding whether currently used canine parvovirus vaccines will provide full protection against the new variant, Asian-CPV-2c. In summary, our results demonstrated the emergence of the new variant Asian-CPV-2c in dogs and cats in Thailand. Since cats can be infected with CPV-2c, dogs can also be infected with FPV. Thus, veterinary practitioners should focus more attention on both CPV and FPV infections, especially interspecies transmission. In Thailand, the surveillance of CPV and FPV should be further conducted on a larger scale to determine the dynamics of predominant variants and their distributions. This information will aid early diagnosis and the development of future strategies for domestic animal vaccination.

## CHAPTER VIII

### Canine rotavirus

Parts of this work have been published in

#### **Molecular characterization identifies intra-host recombination and zoonotic potential of canine rotavirus among dogs from Thailand.**

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#### **8.1 Abstract**

From September 2016 to January 2019, we collected 710 rectal swabs from both healthy and sick dogs from small animal hospitals in 5 provinces of Thailand. The samples were tested for canine rotavirus group A (CRV) by using one-step RT-PCR specific to the VP6 gene. Our results showed that 0.70% (5/710) were positive for CRV. The five CRVs were then characterized by whole-genome sequencing. Our results showed that the genotype of Thai CRVs is G3P[3], which is the predominant genotype reported in dogs. The Thai CRVs posed a novel genetic constellation “G3-P[3]-I3-R3-C3-M3-A9-N2-T3-E3-H6”, which has never been reported in CRVs from dogs but has been reported in rotaviruses from humans. Based on phylogenetic analysis, the Thai CRVs are the result of multiple-reassortments in which gene segments might have originated from human and bat rotaviruses and suggests the zoonotic potential of the virus.

**Keywords:** Canine; Characterization; Rotavirus; Thailand; Zoonotic

## 8.2 Introduction

Rotavirus (RV) is an RNA virus belonging to the *Reoviridae* family. There are nine groups of rotaviruses (A-I). Group A rotavirus (RVA) is one of the major pathogens causing gastroenteritis in humans and animals worldwide (Greenberg and Estes, 2009). The virus contains 11 dsRNA segments encoding viral structure proteins (VP1, VP2, VP3, VP4, VP6 and VP7) and nonstructural proteins (NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6). The RVAs can be classified based on two classification systems. In the first classification system, two outer layer proteins (VP7 and VP4) are used to determine the genotype by G and [P]. There are 35G (G1-G35) and 50P (P[1]-P[50]) (Matthijssens et al., 2011a). Another classification system, the Rotavirus Classification Working Group (RCWG), classifies the rotavirus genotype based on 11 genes as VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6, which presents as the acronym Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx to study evolution, the combination of inter-species transmission and reassortments between human and animal rotaviruses (Doro et al., 2015).

Based on the complete genome analyses of RVAs, the viruses can be classified into three genotypes, genotype 1 (Wa-like), genotype 2 (DS-1like) and genotype 3 (AU-1 like). The RVA genogroups 1 and 2 are the predominant genogroups circulating worldwide. Genotype 3 (AU-1 like) can be further classified into three genogroups; Cat 97-like genogroup (G3-P[3]-I3-R3-C2-M3-A9-N2-T3-E3-H6) (e.g., RO1845, HCR3A) (Tsugawa and Hoshino, 2008), AU-1-like genogroup (G3-P[9]-I3-R3-C3-M3-A3-N3-T3-E3-H3) (e.g., T152) (Rahman et al., 2007), and BA222-05-like genogroup (G3-P[9]-I2-R2-C2-M2-A3-N1/2-T6/3-E2-H3) (e.g., PAI58, PAH136) (Matthijssens et al., 2011b).

In humans, RVAs were first detected in 1973 and have been reported worldwide. RVA causes around 500,000 deaths per year in children, especially in developing countries (Parashar et al., 2009). The common genotypes of RVAs in humans are G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8]. While, G3P[3] and G3P[9] are rare genotypes and cause asymptomatic or mild gastroenteritis in humans (Ro1845, PA260/97 and CU-365). G3P[3] and G3P[9] in humans have been reported in several countries such as Brazil, Japan, India, Italy, Taiwan, and Thailand (Banerjee et al., 2007;



De Grazia et al., 2007a; De Grazia et al., 2007c; Degiuseppe et al., 2015; Khamrin et al., 2006c; Luchs et al., 2012; Okitsu et al., 2018; Theamboonlers et al., 2013b; Tsugawa and Hoshino, 2008).

In dogs, canine rotavirus (CRV) causes moderate to severe gastroenteritis disease (abdominal pain, mucoid diarrhea, and leukocytosis) in puppies younger than 2 weeks (Pollock and Carmichael, 1983). The prevalence of CRV is 2-8% in dogs of a young age with gastroenteritis (Alves et al., 2018; Mochizuki et al., 2001; Ortega et al., 2017b). A previous study reported a high prevalence (80%) of rotavirus antibodies in adult dogs (Rimmelzwaan et al., 1991). Rotavirus genotype G3P[3] is the predominant genotype in dogs in many countries, including Belgium, Japan, Hungary, Italy, South Korea and the United States (Kang et al., 2007; Matthijssens et al., 2011b; Mihalov-Kovacs et al., 2015; Tsugawa and Hoshino, 2008).

Rotavirus is species specific, but cases of cross-species transmission from animals to humans via direct interspecies transmission or reassortment among viruses have been reported. Canine rotavirus might be less virulent in dogs, but it is likely to cause disease in humans including strains Ro1845 and PA260/97 (Tsugawa and Hoshino, 2008; Wu et al., 2012a). It has been reported that the rotavirus genotypes G3P[9] (CU365) and G3P[3] (CMH222), which potentially originated from ruminants, cats, and dogs, could be isolated from children with gastroenteritis in Thailand (Khamrin et al., 2006b; Theamboonlers et al., 2013b). In this study, we surveyed CRVs in dogs in Thailand from September 2016 to January 2019. The Thai CRVs were characterized by whole genome sequencing to determine the genotypes and possible multiple-reassortment of the viruses.

## 8.3 Materials and Methods

### 8.3.1. Sample collection

Sample collection from domestic dogs was conducted in small animal hospitals in 5 provinces of Thailand (Ayutthaya, Bangkok, Suphanburi, Nakhon Ratchasima and Tak) from September 2016 to January 2019. The 710 rectal swab samples were collected from healthy dogs (n = 93) and dogs with gastroenteritis symptoms (n = 617), including vomiting, watery diarrhea, hemorrhagic diarrhea, and dehydration. The swab samples were collected from dogs of a young age (< 1 year) (n = 389) and dogs older than 1 year (>1 year) (n=321). The animals' demographic data, including age, sex, breed, and vaccination history, were also recorded. The study was conducted under the Chulalongkorn University's Animal Use and Care Protocol # 1731074.

### 8.3.2. Canine rotavirus detection

All 710 samples were subjected to canine rotavirus identification by one-step RT-PCR using primers specific to the VP6 gene of CRV. First, RNA extraction was performed using the QIAasympphony DSP Viral/Pathogen mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. To detect CRV, RNA samples were screened for CRV by using a one-step RT-PCR assay with the primers previously described, VP6F (5'-GACGGVGCRACTACATGGT-3') and VP6R (5'-GTCCAATTCATNCCTGGTGG-3') (Ortega et al., 2017b). Briefly, one-step RT-PCR was conducted in a final volume of 50  $\mu$ l comprised of 3  $\mu$ l of template RNA, 25  $\mu$ l of 2xReaction Mix, 1.2  $\mu$ l of 10  $\mu$ M forward and reverse primers, 2.4  $\mu$ l of SuperScript III RT (Invitrogen, CA) and distilled water to a final volume of 50  $\mu$ l. The conditions of the RT-PCR assay included a cDNA synthesis step at 55  $^{\circ}$ C for 30 min, an initial denaturation step at 94  $^{\circ}$ C for 2 min, following 40 cycles of denaturation at 94  $^{\circ}$ C for 30 s, annealing at 52  $^{\circ}$ C for 30 s and extension at 68  $^{\circ}$ C for 1 min, as well as a final extension step at 68  $^{\circ}$ C for 5 min. The expected size of the CRV positive amplified products was 379 bp. Due to the dogs showing clinical signs similar to other canine

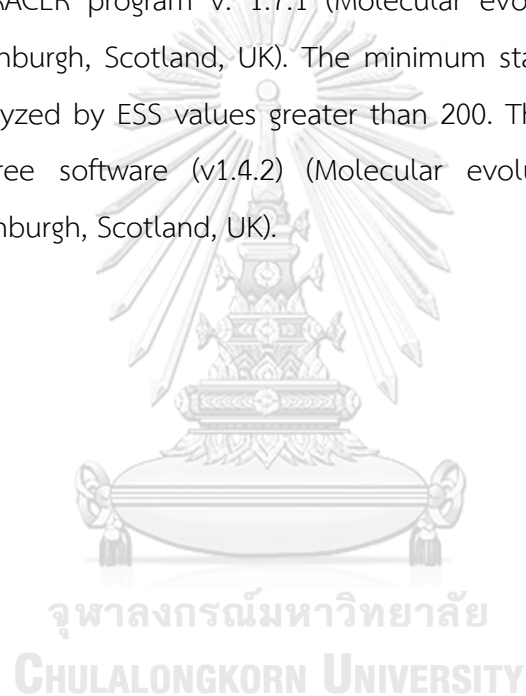
viral enteric diseases, all samples were also tested for Canine Parvovirus, Canine Coronavirus, and Canine Kobuvirus.

### 8.3.3. Canine rotavirus characterization

In this study, five CRV positive samples were subjected to whole-genome sequencing. Each viral gene was amplified using either primers previously described, or newly designed primer sets from the Primer 3 plus program (Koressaar and Remm, 2007b; Tsugawa and Hoshino, 2008). Briefly, one-step RT-PCR was conducted in a final total volume of 50  $\mu$ l comprised of 3  $\mu$ l of template RNA, 25  $\mu$ l of 2xReaction Mix, 1.2  $\mu$ l of 10  $\mu$ M forward and reverse primers, 2.4  $\mu$ l of SuperScript III RT (Invitrogen, CA) and distilled water to a final volume of 50  $\mu$ l. The conditions of the RT-PCR assay included a cDNA synthesis step at 55  $^{\circ}$ C for 30 min, an initial denaturation step at 94  $^{\circ}$ C for 2 min, followed by 40 cycles of denaturation at 94  $^{\circ}$ C for 30 s, annealing at 48-55  $^{\circ}$ C for 30 s and extension at 68  $^{\circ}$ C for 1-4 min, as well as a final extension step at 68  $^{\circ}$ C for 5 min. PCR products were purified by NucleoSpin® Gel and PCR Clean-up (MACHEREY-NAGEL™, Germany). The purified PCR products were submitted to NovogeneAIT (Singapore) for HiSeq Illumina paired end 50 cycles sequencing by using a NEBNext® Ultra™ DNA kit for library preparation. The nucleotide sequences were assembled by trimmed-reads and by mapping all reads to the reference. Whole genome sequences and the partial genome were extracted using a CLC genomic Workbench module 11.0 (Qiagen, Hilden, Germany).

Phylogenetic and genetic analyses were performed by comparing each gene of the CRV to the reference CRV and the other RV sequences available in the GenBank database. The reference nucleotide sequences included in the RV represent various geographical origins, host origins, and date of isolations, and followed the previous studies (Grant et al., 2011; Okitsu et al., 2018; Sasaki et al., 2016; Theamboonlers et al., 2013b). It should be noted that only seven complete genome sequences of CRV have been reported and included as reference CRVs in the databases (RV52-96/Italy, RV198-95/ Italy, CU only 7 strains CRV (RV52-96/Italy, RV198-95/Italy, CU-1/USA, K9/USA, A79-10/USA, RS15/Japan, and HUN135/Hungary). Then, the nucleotide sequences of each gene were aligned by using the Muscle

program v.3.6. The evolution analysis was performed by using a BEAST 1.8 program applying a Bayesian Markov Chain Monte Carlo (BMCMC) algorithm. The best-fit substitution model was implemented by MEGA 7. A strict clock model with coalescent constant population and GTR, TN93 (G+I) and HKY with gamma 4 substitution were used as model parameters. The Bayesian MCMC chain lengths were 10,000,000–80,000,000 generations, with sampling every 10,000 generations. The tree iteration was discharged with 10% of the chains as a burn-in pattern by using a tree annotator. The parameters were confirmed by calculating the Effective Sample Size (ESS) with the TRACER program v. 1.7.1 (Molecular evolution, phylogenetics and epidemiology, Edinburgh, Scotland, UK). The minimum standard error in each gene segment was analyzed by ESS values greater than 200. The resulting MCC tree was drawn with FigTree software (v1.4.2) (Molecular evolution, phylogenetics and epidemiology, Edinburgh, Scotland, UK).



## 8.4 Results

### 8.4.1. Canine rotavirus in dogs in Thailand

From September 2016 to January 2019, we collected 710 rectal swabs from both healthy and sick dogs from small animal hospitals in 5 provinces of Thailand. All rectal swab samples were tested for canine rotavirus group A by using one-step RT-PCR specific to the VP6 gene. Our results showed that 0.70% (5/710) were positive for CRV. RVA could be detected from both sick and asymptomatic dogs. The positive samples were all collected from young dogs of age <1 year. All five CRVs were then subjected to whole-genome sequencing and the nucleotide sequences of the Thai CRVs were submitted to the GenBank database under the accession number MT364824-78 (Table 8.1 and Table 8.2).

### 8.4.2. Genotype and the genetic constellation of Thai canine rotaviruses

In this study, five Thai CRVs designated RVA/Dog-wt/THA/CU126/2017/G3P[3] (CU126), RVA/Dog-wt/THA/CU128/2017/G3P[3] (CU128), RVA/Dog-wt/THA/CU132/2017/G3P[3] (CU132), RVA/Dog-wt/THA/CU20139/2017/G3P[3] (CU20139) and RVA/Dog-wt/THA/CU23379/2019/G3P[3] (CU23379) were characterized. The genotype of the CRVs was identified by the RotaC program (<http://rotac.regatools.be/>). The genetic constellation of five Thai CRVs was G3-P[3]-I3-R3-C3- M3-A9-N2-T3-E3-H6. Then, we compared the genetic constellation of the Thai CRVs to the reference RVs from cats, dogs, bats, pigs, horses and humans. Our results showed that the Thai CRVs were identical to Human RVA in 2014 (RVA/Human-wt/JPN/12638/2014/G3P[3]), which has the motif G3-P[3]-I3-R3-C3- M3-A9-N2-T3-E3-H6 (nucleotide identities ranging from 92.90-99.20%). It should be noted that the Thai CRVs belong to genotype AU-1 with gene segments of both genogroup Au-1-like and Cat 97-like), which have never been reported before for any canine rotaviruses (Table 8.3 and Table 8.4).

**Table 8.1.** Description of the Thai CRVs characterized in this study.

Viruses	Date	location	region	Age	Breed	Clinical signs	Accession number
RVA/Dog-wt/THA/CU126/2017/G3P[3]	Jan-2017	Tak	Northern	2 mts	Mixed	Asymptomatic	MT364824-34
RVA/Dog-wt/THA/CU128/2017/G3P[3]	Jan-2017	Tak	Northern	2 mts	Mixed	Asymptomatic	MT364835-45
RVA/Dog-wt/THA/CU132/2017/G3P[3]	Jan-2017	Tak	Northern	2 mts	Mixed	Asymptomatic	MT364846-56
RVA/Dog-wt/THA/CU20139/2017/G3P[3]	Nov-2017	Bangkok	Central	2 mts	Beagle	Diarrhea	MT364857-67
RVA/Dog-wt/THA/CU23379/2019/G3P[3]	Jan-2019	Bangkok	Central	2 mts	German shepherd	Diarrhea	MT364868-78

**Table 8.2.** Association between age and clinical presentations of CRVs detection in this study

Age	Canine rotavirus positives (%)	
	Asymptomatic	Clinical sign
Young (<1 year)	3/12 (25%)	2/377 (0.53%)
Adult (1-5 years)	0/78 (0%)	0/137 (0%)
Older (>5 years)	0/3 (0%)	0/103 (0%)
	<b>3/93 (3.23%)</b>	<b>2/617 (0.32%)</b>

**Table 8. 3.** The genetic constellation of the Thai CRVs and reference rotaviruses

Virus	Genotype <sup>a</sup>	Genogroup <sup>b</sup>	Gene										
			VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
<b>This study</b>													
RVA/Dog/THA/CU126/2017/G3P[3]	AU-1	AU-1-like/ Cat97-like	G3	P[3]	I3	R3	C3	M3	A9	N2	T3	E3	H6
RVA/Dog/THA/CU 128/2017/G3P[3]	AU-1	AU-1-like/ Cat97-like	G3	P[3]	I3	R3	C3	M3	A9	N2	T3	E3	H6
RVA/Dog/THA/CU 132/2017/G3P[3]	AU-1	AU-1-like/ Cat97-like	G3	P[3]	I3	R3	C3	M3	A9	N2	T3	E3	H6
RVA/Dog/THA/CU 20139/2017/G3P[3]	AU-1	AU-1-like/ Cat97-like	G3	P[3]	I3	R3	C3	M3	A9	N2	T3	E3	H6
RVA/Dog/THA/CU 23379/2019/G3P[3]	AU-1	AU-1-like/ Cat97-like	G3	P[3]	I3	R3	C3	M3	A9	N2	T3	E3	H6
<b>Canine</b>													
RVA/Dog-tc/ITA/RV52-96/1996/G3P[3]	AU-1	Cat 97-like	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Dog-tc/ITA/RV198-95/1995/G3P3	AU-1	Cat 97-like	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Dog-tc/USA/CU-1/1982/G3P[3]	AU-1	Cat 97-like	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Dog-tc/USA/K9/1981/G3P[3]	AU-1	Cat 97-like	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Dog-tc/USA/A79-10/1979/G3P[3]	AU-1	Cat 97-like	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Dog-tc/JPN/RS15/1982/G3P[3]	AU-1	Cat 97-like	G3	P[3]	I3	R3	C2	M3	A9	N3	T3	E3	H6
<b>Feline</b>													
RVA/Cat-tc/AUS/Cat97/1984/G3P[3]	AU-1	Cat 97-like	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Cat-wt/ITA/BA222/2005/G3P[9]	AU-1	BA222-05-like	G3	P[9]	I2	R2	C2	M2	A3	N1	T3	E2	H3
<b>Bat</b>													
RVA/Bat-wt/ZMB/LUS12-14/2012/G3P[3]	AU-1	Cat 97 /BA222-05-like	G3	P[3]	I3	R2	C2	M3	A9	N2	T3	E2	H3
<b>Human</b>													
RVA/Human-tc/JPN/AU-1/1982/G3P[9]	AU-1	AU-1 like	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
RVA/Human-tc/THA/T152/1998/G12P[9]	AU-1	AU-1 like	G12	P[9]	I3	R3	C3	M3	A12	N3	T3	E3	H6
RVA/Human-tc/CHN/L621/2006/G3P[9]	AU-1	AU-1 like	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H6
RVA/Human-wt/CHN/E2451/2011/G3P[9]	AU-1	AU-1 like	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H6
RVA/Human-tc/THA/CU-365/2008/G3P[9]	AU-1	AU-1 like	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H6
RVA/Human-wt/ITA/PAH136/1996/G3P[9]	AU-1	BA222-05-like	G3	P[9]	I2	R2	C2	M2	A3	N1	T6	E2	H3
RVA/Human-wt/ITA/PA158/1996/G3P[9]	AU-1	BA222-05-like	G3	P[9]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Human-tc/USA/HCR3A/1984/G3P[3]	AU-1	Cat 97-like	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Human-tc/ISR/Ro1845/1985/G3P[3]	AU-1	Cat 97-like	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Human-tc/ITA/PA260-97/1997/G3P[3]	AU-1	AU-1-like/ Cat97-like	G3	P[3]	I3	R3	C3	M3	A15	N2	T3	E3	H6
RVA/Human-wt/JPN/12638/2014/G3P[3]	AU-1	AU-1-like /Cat97-like	G3	P[3]	I3	R3	C3	M3	A9	N2	T3	E3	H6
RVA/Human-wt/THA/CMH222/2001/G3P[3]	AU-1	N/A	G3	P[3]	I8	-	-	-	-	-	-	E3	-
RVA/Human-tc/USA/Wa/1974/G1P[8]	Wa	N/A	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-tc/USA/DS-1/1976/G2P[4]	DS-1	N/A	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2

<sup>a</sup> RVA genotype based on a classification system of 11 gene segments can be classified into three genotypes; AU-1, Wa, and DS-1

<sup>b</sup> AU-1 genotype can be classified into three genogroups; Au-1-like, Cat 97-like and BA222-05

Table 8.4. Pair-wise comparison among genes of Thai CRVs and reference rotaviruses.

Virus	Gene										
	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
<b>Nucleotide (bp)/amino acid length (aa)</b>	981 bp (327 aa)	2328bp (726aa)	1194bp (398aa)	3267bp (1089aa)	2664bp (888aa)	2508bp (836aa)	1482bp (494aa)	954bp (318aa)	933bp (311aa)	528bp (176aa)	597bp (199aa)
<b>RVA/Dog/THA/CU 20139/2017/G3P[3]</b>	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)	100.00 (100.00)
<b>RVA/Dog/THA/CU126/2017/G3P[3]</b>	98.80 (100.00)	98.40 (98.90)	98.60 (100.00)	98.70 (99.80)	97.20 (99.60)	93.40 (96.80)	98.20 (99.20)	98.70 (99.40)	91.50 (94.00)	99.00 (98.80)	98.70 (98.50)
<b>RVA/Dog/THA/CU 128/2017/G3P[3]</b>	98.80 (100.00)	98.40 (98.90)	98.60 (100.00)	98.70 (99.80)	97.20 (99.60)	93.40 (96.80)	98.20 (99.20)	98.70 (99.40)	91.50 (94.00)	99.00 (98.80)	98.70 (98.50)
<b>RVA/Dog/THA/CU 132/2017/G3P[3]</b>	98.80 (100.00)	98.40 (98.90)	98.60 (100.00)	98.70 (99.80)	97.20 (99.60)	93.40 (96.90)	98.20 (99.20)	98.70 (99.40)	91.50 (94.00)	99.00 (98.80)	98.70 (98.50)
<b>RVA/Dog/THA/CU 23379/2017/G3P[3]</b>	98.00 (99.30)	98.10 (98.80)	99.40 (99.50)	98.40 (99.30) *****	96.70 (99.90)	93.30 (96.90)	97.80 (98.30)	98.40 (99.70)	91.50 (94.90)	98.80 (99.40)	98.50 (98.50)
<b>Canine</b>											
<b>RVA/Dog-tc/ITA/RV52-96/1996/G3P[3]</b>	82.80 (94.30)	96.30 (96.70)	96.60 (99.00)	86.40 (96.10)	82.60 (96.80)	94.70 (97.60)	82.80 (86.00)	97.60 (98.70)	85.80 (90.80)	98.30 (98.80)	93.20 (94.50)
<b>RVA/Dog-tc/ITA/RV198-95/1995/G3P[3]</b>	93.70 (98.60)	94.20 (96.50)	97.30 (99.50)	86.30 (96.00)	82.30 (97.40)	94.40 (97.20)	83.30 (86.80)	97.20 (98.40)	86.30 (91.10)	85.00 (93.10)	93.80 (94.50)
<b>RVA/Dog-tc/USA/CU-1/1982/G3P[3]</b>	95.60 (98.60)	93.40 (96.10)	84.80 (96.90)	86.50 (96.40)	82.20 (97.00)	84.60 (91.70)	83.00 (86.80)	84.80 (93.30)	84.70 (90.50)	83.20 (95.40)	93.80 (94.00)
<b>RVA/Dog-tc/USA/K9/1981/G3P[3]</b>	94.00 (98.20)	94.90 (96.60)	84.50 (96.60)	86.10 (96.20)	82.20 (97.30)	84.70 (92.00)	83.00 (86.20)	85.30 (92.90)	84.70 (91.10)	84.80 (93.10)	92.70 (93.00)
<b>RVA/Dog-tc/USA/A79-10/1979/G3P[3]</b>	95.70 (98.60)	93.70 (97.00)	84.90 (96.60)	86.60 (96.50)	82.30 (97.30)	84.20 (91.60)	83.60 (86.80)	84.60 (92.90)	85.10 (91.10)	84.60 (93.10)	93.30 (94.00)
<b>RVA/Dog-tc/JPN/RS15/1982/G3P[3]</b>	93.40 (98.60)	95.20 (96.90)	85.30 (97.90)	86.40 (96.60)	82.40 (97.30)	84.40 (91.60)	82.80 (86.40)	80.20 (88.10)	85.30 (91.70)	92.30 (95.40)	94.00 (94.00)
<b>RVA/Dog-wt/HUN/135/2012/G3P[3]</b>	82.60 (93.50)	95.90 (96.90)	96.80 (99.00)	93.50 (97.60)	94.60 (99.30)	94.40 (97.70)	49.30 (38.10)	97.00 (99.00)	84.90 (90.80)	97.70 (97.70)	93.50 (94.50)
<b>Feline</b>											
<b>RVA/Cat-tc/AUS/Cat97/1984/G3P[3]</b>	93.40 (98.60)	93.70 (96.30)	84.70 (96.60)	86.40 (96.20)	82.40 (97.30)	84.20 (92.10)	83.00 (86.20)	84.90 (92.90)	85.50 (91.40)	85.20 (93.10)	93.70 (93.50)
<b>RVA/Cat-tc/AUS/Cat2/1984/G3P[9]</b>	80.50 (92.10)	66.20 (68.90)	86.00 (97.90)	86.40 (96.60)	82.30 (97.50)	84.20 (91.60)	47.40 (35.70)	82.70 (88.50)	76.20 (80.00)	85.40 (93.10)	90.00 (92.20)
<b>RVA/Cat/JPN/FRV348/1994/G3P[3]</b>	83.50 (95.00)	96.90 (97.60)	97.50 (99.50)	93.90 (97.80)	96.30 (99.60)	94.60 (97.30)	49.90 (39.90)	80.40 (88.10)	96.50 (97.50)	97.70 (97.70)	93.60 (94.40)
<b>RVA/Cat/JPN/FRV384/1994/G3P[9]</b>	79.50 (92.10)	66.20 (69.20)	86.30 (97.70)	87.40 (97.00)	88.10 (99.30)	87.00 (92.90)	48.00 (36.70)	79.00 (86.20)	84.60 (92.10)	90.40 (94.80)	88.70 (90.00)
<b>RVA/Cat-wt/ITA/BA222/2005/G3P[9]</b>	79.80 (91.00)	66.50 (69.20)	82.90 (95.80)	79.20 (93.90)	81.50 (97.00)	76.60 (83.90)	47.70 (36.10)	81.80 (89.70)	85.10 (92.40)	77.60 (83.80)	89.20 (90.00)
<b>Horse</b>											
<b>RVA/Horse/ARG/E3198/2008/G3P[3]</b>	84.00 (96.10)	81.20 (90.80)	85.30 (97.90)	85.00 (93.30)	88.60 (99.50)	87.20 (94.50)	87.80 (91.10)	80.40 (88.50)	88.80 (93.30)	89.20 (94.20)	96.50 (97.00)
<b>RVA/Horse-wt/ARG/E30/1993/G3P[12]</b>	87.90 (93.20)	74.70 (82.30)	81.10 (92.50)	73.80 (81.90)	81.00 (96.10)	83.00 (88.20)	73.10 (74.00)	88.10 (96.20)	87.20 (92.10)	78.80 (81.50)	82.80 (86.50)



Table 8.4. Pair-wise comparison among genes of Thai CRVs and reference rotaviruses.

(cont.)

Virus	Gene										
	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
<b>Nucleotide (bp)/amino acid length (aa)</b>	981 bp (327 aa)	2328bp (726aa)	1194bp (398aa)	3267bp (1089aa)	2664bp (888aa)	2508bp (836aa)	1482bp (494aa)	954bp (318aa)	933bp (311aa)	528bp (176aa)	597bp (199aa)
<b>Bat</b>											
RVA/Bat-wt/ZMB/LUS12-14/2012/G3P[3]	83.10 (94.60)	97.30 (98.00)	94.60 (99.20)	78.50 (88.10)	82.10 (97.40)	93.70 (96.90)	98.10 (98.60)	98.30 (98.40)	91.00 (94.30)	77.80 (82.70)	88.80 (91.00)
RVA/Bat-wt/CHN/MSLH14/2012/G3P[3]	84.80 (95.00)	80.90 (91.20)	82.20 (96.60)	79.30 (90.80)	87.60 (99.50)	87.10 (94.70)	86.80 (92.40)	80.10 (88.50)	87.40 (91.40)	88.40 (94.20)	93.20 (96.50)
RVA/Bat-wt/CHN/LZHP2/2015/G3P[3]	84.20 (95.30)	80.70 (88.30)	85.60 (97.40)	79.90 (94.00)	87.50 (96.20)	87.40 (92.40)	87.10 (90.50)	81.10 (88.10)	85.30 (90.80)	91.70 (94.80)	92.80 (95.50)
RVA/Bat-wt/CMR/BatLi09/2014/G30P[42]	70.80 (72.80)	70.00 (73.80)	75.60 (82.60)	84.70 (96.50)	79.40 (90.90)	67.40 (66.40)	36.70 (22.30)	72.20 (72.10)	71.20 (73.10)	61.90 (64.20)	69.00 (67.30)
<b>Swine</b>											
RVA/Pig-tc/VEN/A131/1988/G3P[7]	81.30 (90.30)	N/A	77.60 (89.90)	89.00 (97.50)	75.50 (85.10)	76.60 (80.10)	46.10 (33.80)	79.60 (83.70)	72.00 (74.90)	75.10 (83.80)	84.30 (84.50)
RVA/Pig-tc/ESP/OSU-C5111/2010/GP[7]	28.90 (10.50)	74.50 (80.60)	79.00 (90.40)	80.60 (93.60)	80.90 (94.50)	77.70 (83.70)	47.50 (35.30)	31.80 (10.30)	76.10 (82.90)	77.60 (82.70)	90.20 (92.20)
<b>Human</b>											
RVA/Human-tc/USA/Wa/1974/G1P[8]	72.90 (80.30)	68.60 (70.70)	79.80 (92.50)	79.40 (90.90)	80.80 (94.00)	77.80 (83.60)	48.00 (36.70)	81.10 (89.10)	78.20 (80.30)	81.10 (85.50)	87.70 (89.50)
RVA/Human-tc/USA/DS-1/1976/G2P[4]	70.00 (71.70)	69.20 (71.20)	82.40 (94.80)	80.20 (93.80)	81.30 (96.50)	76.40 (83.40)	48.70 (34.20)	87.00 (94.20)	80.70 (87.30)	78.40 (83.20)	83.50 (83.50)
RVA/Human-tc/JPN/AU-1/1982/G3P[9]	79.80 (91.40)	66.30 (69.20)	84.90 (97.90)	93.10 (98.20)	93.40 (99.30)	87.60 (94.00)	46.90 (35.30)	79.80 (87.80)	84.80 (92.10)	90.20 (94.20)	88.30 (90.00)
RVA/Human-tc/THA/T152/1998/G12P[9]	73.20 (80.30)	67.20 (69.60)	88.70 (99.00)	96.20 (98.90)	95.90 (99.50)	93.80 (96.20)	49.90 (37.50)	79.60 (87.50)	96.70 (97.10)	91.30 (94.80)	94.30 (96.00)
RVA/Human-tc/CHN/L621/2006/G3P[9]	80.30 (91.40)	66.10 (69.00)	86.50 (98.20)	86.40 (96.30)	98.80 (100.00)	87.50 (93.90)	47.30 (35.50)	79.60 (86.50)	89.10 (94.30)	89.20 (93.60)	95.80 (95.50)
RVA/Human-wt/ITA/PAH136/1996/G3P[9]	79.60 (90.7)	66.40 (69.30)	81.70 (95.80)	80.30 (94.30)	81.70 (97.20)	76.40 (83.80)	47.70 (35.30)	82.40 (88.50)	77.60 (79.40)	78.20 (82.70)	88.50 (90.00)
RVA/Human-wt/CHN/E2451/2011/G3P[9]	80.80 (91.80)	66.10 (68.90)	85.70 (98.20)	86.30 (95.90)	94.90 (98.80)	87.30 (93.90)	47.60 (36.10)	79.50 (85.90)	84.40 (91.10)	89.40 (93.60)	95.50 (95.50)
RVA/Human-tc/THA/CU-365/2008/G3P[9]	79.80 (92.10)	66.00 (69.20)	86.20 (98.20)	95.80 (98.50)	88.20 (99.20)	97.60 (98.10)	47.80 (35.30)	79.20 (85.90)	88.70 (94.90)	88.80 (92.50)	95.50 (94.50)
RVA/Human-tc/ITA/PA260-97/1997/G3P[3]	82.30 (93.50)	96.70 (97.10)	97.30 (99.50)	93.80 (98.00)	95.00 (99.50)	94.40 (97.20)	49.60 (38.40)	97.80 (99.00)	91.30 (94.00)	97.70 (98.80)	93.80 (95.00)
RVA/Human-wt/THA/CMH222/2001/G3P[3]	83.80 (95.30)	80.30 (89.80)	82.60 (96.10)	N/A	N/A	N/A	N/A	N/A	N/A	88.10 (95.40)	N/A
RVA/Human-wt/ITA/PA158/1996/G3P[9]	80.70 (92.10)	66.40 (68.90)	82.30 (96.10)	79.70 (94.40)	80.80 (97.00)	76.90 (84.00)	48.40 (35.90)	91.70 (95.80)	84.90 (90.50)	77.30 (83.80)	88.70 (91.50)
RVA/Human-wt/JPN/12638/2014/G3P[3]	98.80 (99.30)	92.90 (96.10)	99.20 (99.50)	97.40 (99.40)	98.80 (100.00)	98.50 (98.60)	98.00 (98.40)	97.90 (98.40)	91.30 (95.20)	97.70 (98.80)	97.70 (98.00)
RVA/Human-tc/USA/HCR3A/1984/G3P[3]	95.10 (98.90)	94.20 (95.70)	84.70 (96.90)	86.10 (96.50)	82.30 (97.40)	84.80 (92.20)	83.20 (86.60)	85.00 (93.30)	84.60 (91.10)	84.00 (92.50)	93.50 (93.50)
RVA/Human-tc/ISR/Ro1845/1985/G3P[3]	93.80 (98.60)	93.90 (96.30)	85.10 (96.90)	86.10 (96.70)	82.20 (97.50)	84.20 (91.50)	82.90 (86.40)	85.40 (93.30)	85.40 (92.40)	85.40 (93.10)	93.50 (93.50)
RVA/Human-wt/BRA/1A3739/2011/G3P9	82.10 (94.40)	63.80* (62.10)	80.20 (91.70)	86.80** (95.90)	88.60*** (97.70)	N/A	48.30 (34.50)	80.80 (88.50)	86.90 (94.20)	87.90 (94.20)	92.80 (95.50)
RVA/Human/BRA/R2638/2011/G3P[3]	97.00 (98.90)	93.00**** (93.90)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

\* Comparison with 792 bp

\*\*comparison with 590 bp

\*\*\* Comparison with 525 bp

\*\*\*\* Comparison with 739 bp

\*\*\*\*\* Comparison with 1,608bp

### 8.4.3. Genotype G3P[3] of the Thai Canine Rotaviruses

Based on nucleotide identities and phylogenetic analysis of the VP7 and VP4 genes, our results showed that the Thai CRVs belonged to the genotype G3P[3]. For the VP7 gene, the Thai CRVs (n=5) possessed high nucleotide identities to human RVA (12638/Japan) at 98.80% (99.30% aa identities). The nucleotide identities of the VP7 gene of the Thai CRVs compared to that of the other RVAs of the G3 genotype from horses, bats, pigs, cats, and dogs ranged from 79.50 to 95.70% (92.10%-98.60% aa identities).

The phylogenetic tree of the VP7 of the RVAs showed that genotype G3 could be divided into 2 major clusters, A and B, and subclusters b1 and b2. The Thai CRVs were grouped with subcluster b2 and were closely related to human RVA (12638/Japan, R2638/Brazil and HCR3A/USA). Based on the MCC phylogenetic tree, the Thai CRVs were estimated to have separated from human rotavirus (R2638/Brazil) since 1989 (Table 8.5 and Figure 8.1). The estimated nucleotide substitution rate of VP7 was  $5.1127 \times 10^{-4}$  substitutions per site per year (95% posterior densities (HPD);  $3.343 \times 10^{-4}$  -  $6.9163 \times 10^{-4}$ ). For the VP4 gene, the Thai CRVs had highest nucleotide identities to bat RVA (LUS12-14/Zambia) at 97.30% nt identities (98.00% aa identities). Comparing to the other P[3] RVs of horses, bats, dogs, cats and humans, the Thai CRVs had 80.70%-96.90% nt identities (88.30% -97.60% aa identities). The phylogenetic tree of VP4 showed that the Thai CRVs were grouped into the P[3] cluster. MCC tree analysis showed that the Thai CRVs likely diverged from bat RVA (LUS12-14/Zambia) since 1991. The estimated nucleotide substitution rate of VP4 was  $4.5008 \times 10^{-4}$  substitutions per site per year (95% posterior densities (HPD);  $3.4495 \times 10^{-4}$  -  $5.537 \times 10^{-4}$ ) (Table 8.5 and Figure 8.2).

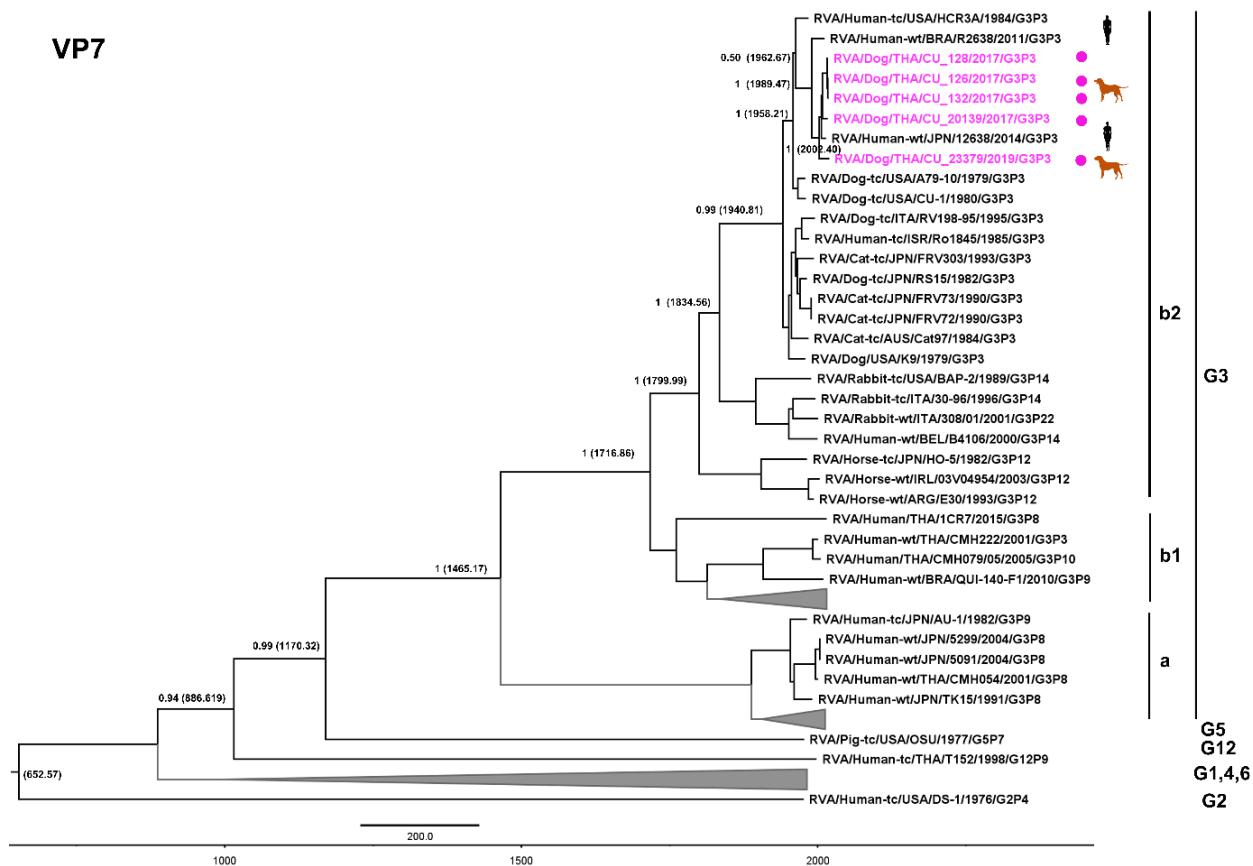
Phylogenetic analysis of the other structural proteins (VP) and nonstructural proteins (NSP) are shown in Table 8.5 and the Figures 8.4-8.11. For VP1, VP2 and VP6, the Thai CRVs possessed the highest nucleotide identities to human rotavirus (12638/JPN). The phylogenetic trees showed that the Thai CRVs were grouped into the R3, C3, and I3 groups. The tMRCA showed that the VP1, VP2 and VP6 of the Thai CRVs were estimated to have separated from human rotavirus (12638/Japan) in the 1990s. The VP3 gene of the Thai CRVs (CU20139) had the highest nucleotide

identities with human RVA (12638/Japan). The Thai CRVs (CU126, CU128, CU132 and CU 23379) had the highest nucleotide identities with bat RVA (LUS12-14/Zambia). The tMRCA revealed that the Thai CRVs (CU126, CU128, CU132 and CU 23379) had diverged from bat RVA (LUS12-14/Zambia) in 1966 and the Thai CRV (CU20139) from human RVA (12638/Japan) in 1997 (Table 8.5, Figure 8.3 -8.6).

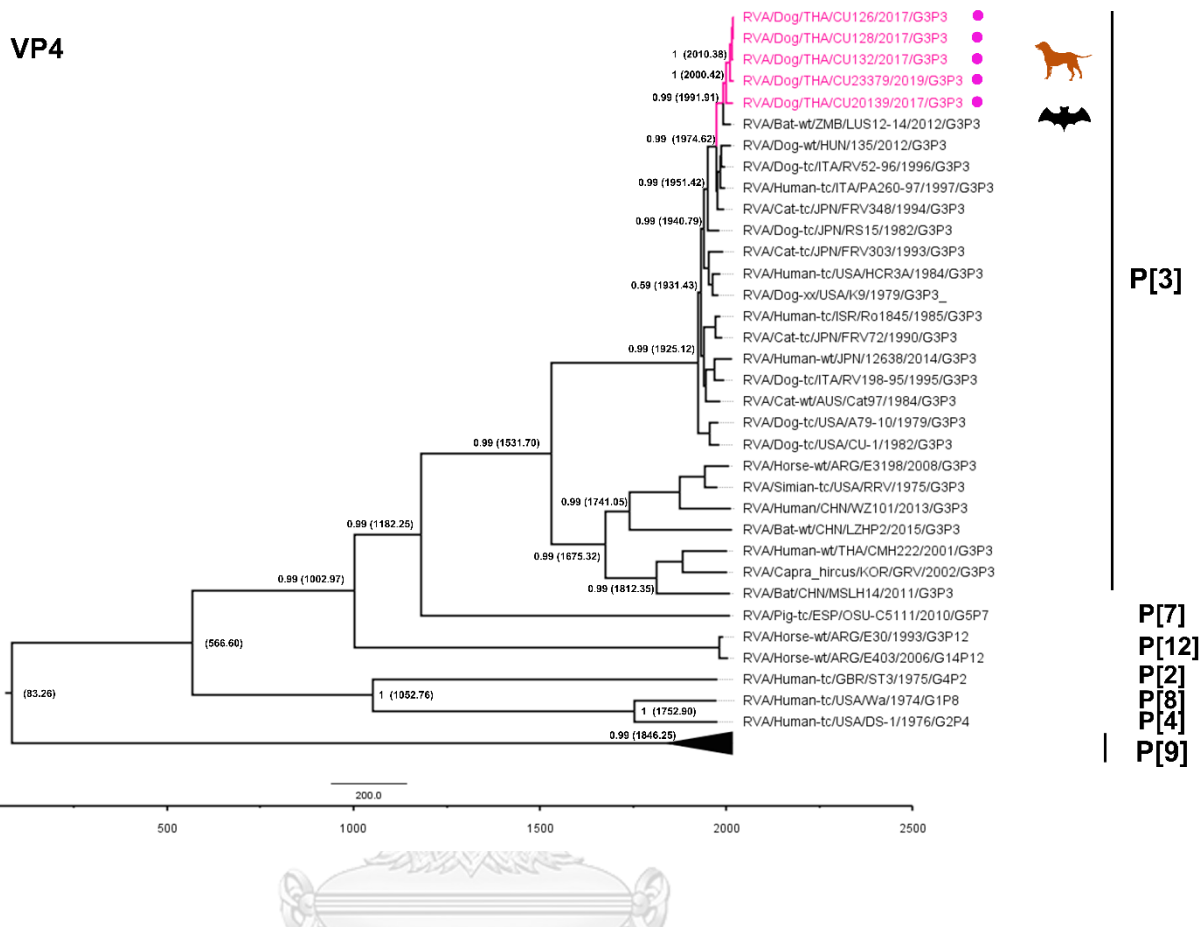
For NSP1 and NSP2, the Thai CRVs had the highest nucleotide identities with a bat RVA (LUS12-14/Zambia). The MCC tree showed that the Thai CRVs were grouped into A9 and N2 groups and originated from bat RVA (LUS12-14/Zambia) and human RVA (12638/Japan) during 1985-1995. For NSP3, the Thai CRV (CU20139) had the highest nucleotide identities with cat RVA (FRV348/JPN). The other Thai CRVs (CU126, CU128, CU132 and CU23379) had the highest nucleotide identities with human RVA (12638/Japan). The MCC tree of NSP3 showed that the Thai CRV (CU20139) was diverged from the cat RVA (strain FRV348/Japan) since 1965. The other Thai CRVs (CU126, CU128, CU132 and CU23379) were likely diverged from human RVA (12638/Japan) in 2002. For NSP4 and NSP5, the Thai CRVs had the highest nucleotide identities with human RVA (12638/Japan). The MCC tree of NSP4 and NSP5 showed that the Thai CRVs were grouped into the E3 and H6 groups and likely diverged from human RVA (12638/Japan) since 1988-1992 (Table 8.5 and Figures 8.7-8.11).

**Table 8.5.** Nucleotide identities, genotype, nucleotide substitution rate and 95% posterior densities of each gene of the Thai CRVs

Gene	Genotype <sup>a</sup>	Closest RVs <sup>b</sup>	% nt identities <sup>b</sup> (% aa identities)	Nucleotide substitution rate/site/year	95% posterior densities (HPD)	Potential origin at tMRCA <sup>c</sup>	tMRCA <sup>c</sup>
VP7	G3	Human RVA (12638/Japan)	98.80% (99.30%)	$5.1127 \times 10^{-4}$	$3.343 \times 10^{-4}$ - $6.9163 \times 10^{-4}$	Human RVA (R2638/Brazil)	1989
VP4	P[3]	Bat RVA (LUS1214/Zambia)	97.30% (98.00%)	$4.5008 \times 10^{-4}$	$3.4495 \times 10^{-4}$ - $5.537 \times 10^{-4}$	Bat RVA (LUS1214/Zambia)	1991
VP1	R3	Human RVA (12638/Japan)	97.40% (99.40%)	$4.63517 \times 10^{-4}$	$3.5527 \times 10^{-4}$ - $5.174 \times 10^{-4}$	Human RVA (12638/Japan)	1994
VP2	C3	Human RVA (12638/Japan)	98.80% (100%)	$2.3015 \times 10^{-4}$	$1.4649 \times 10^{-4}$ - $3.0954 \times 10^{-4}$	Human RVA (12638/Japan; L621/China)	1995
VP3	M3	Human RVA* (12638/Japan)	98.50% (98.60%)	$3.2645 \times 10^{-4}$	$2.225 \times 10^{-4}$ - $4.2199 \times 10^{-4}$	Human RVA (12638/Japan)	1997
		Bat RVA** (LUS1214/Zambia)	98.80% (99.20%)			Bat RVA (LUS1214/Zambia)	1966
VP6	I3	Human RVA (12638/Japan)	99.20% (99.50%)	$3.6436 \times 10^{-4}$	$2.186 \times 10^{-4}$ - $5.1202 \times 10^{-4}$	Human RVA (12638/Japan)	2000
NSP1	A9	Bat RVA (LUS1214/Zambia)	98.10% (98.60%)	$3.0709 \times 10^{-4}$	$1.5829 \times 10^{-4}$ - $4.6072 \times 10^{-4}$	Bat RVA (LUS1214/Zambia)	1985
NSP2	N2	Bat RVA (LUS1214/Zambia)	98.30% (98.40%)	$5.3834 \times 10^{-4}$	$3.766 \times 10^{-4}$ - $7.0075 \times 10^{-4}$	Bat RVA (LUS1214/Zambia)	1995
						Human RVA (12638/Japan)	
NSP3	T3	Cat RVA*** (FRV348/Japan)	96.70% (97.50%)	$4.5655 \times 10^{-4}$	$3.0257 \times 10^{-4}$ - $6.1026 \times 10^{-4}$	Cat RVA (FRV348/Japan)	1965
		Human RVA**** (12638/Japan)	99.00% (99.40%)			Human RVA (12638/Japan)	2002
NSP4	E3	Human RVA (12638/Japan)	97.70% (98.80%)	$5.2603 \times 10^{-4}$	$2.6643 \times 10^{-4}$ - $8.0098 \times 10^{-4}$	Human RVA (12638/Japan)	1992
NSP5	H6	Human RVA (12638/Japan)	97.70% (98.00%)	$4.1703 \times 10^{-4}$	$2.7526 \times 10^{-5}$ - $6.455 \times 10^{-4}$	Human RVA (12638/Japan)	1988

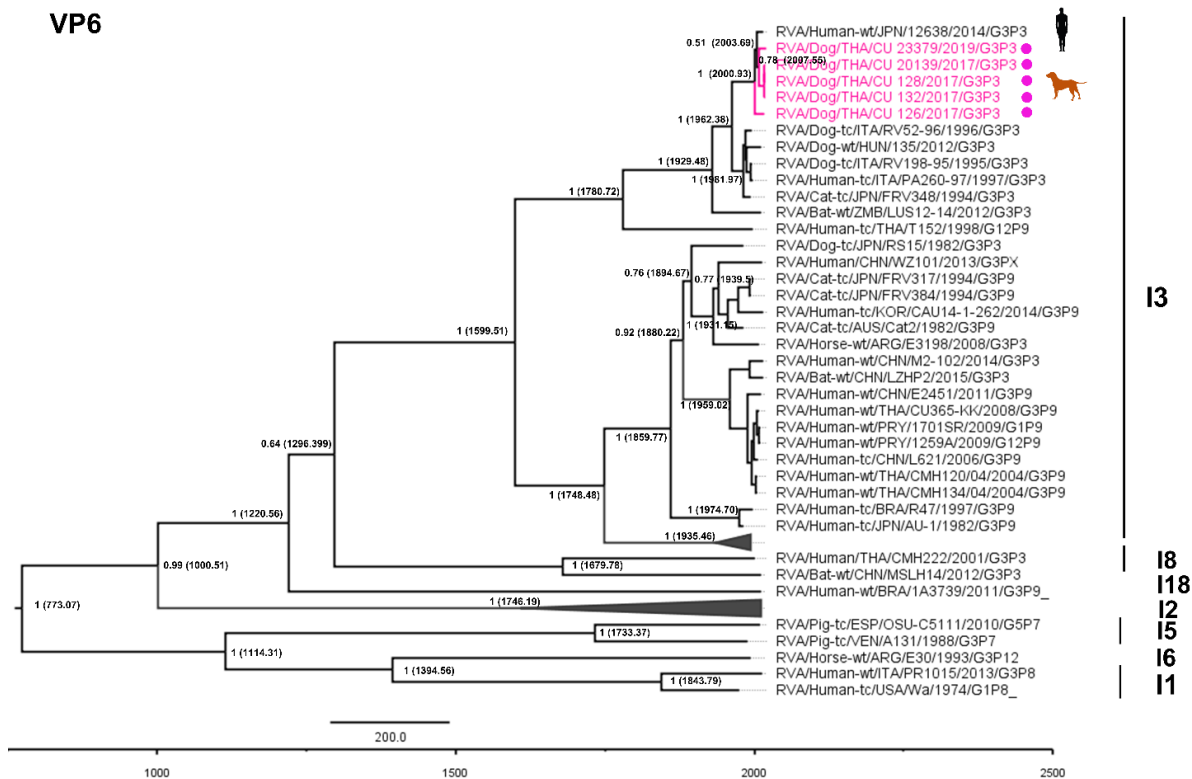


**Figure 8.1.** Phylogenetic tree of VP7. The maximum clade credibility (MCC) tree was constructed by BEAST 1.8 with the Bayesian Markov Chain Monte Carlo (BMCMC) algorithm. A strict clock model with a coalescent constant population and GTR, TN93 (G+I) and HKY with a gamma 4 substitution were used as model parameters. Pink circles indicate the Thai CRVs characterized in this study. Dog images (orange) represent the Thai CRVs and human images (black) represent reference human RVs closely related to the Thai CRVs.



**Figure 8.2.** Phylogenetic tree of VP4. The maximum clade credibility (MCC) tree was constructed by BEAST 1.8 with the Bayesian Markov Chain Monte Carlo (BMCMC) algorithm. A strict clock model with a coalescent constant population and GTR, TN93 (G + I) and HKY with a gamma 4 substitution were used as model parameters. Pink circles indicate the Thai CRVs characterized in this study. Dog images (orange) represent the Thai CRVs and bat images (black) represent the reference bat RVs closely related to the Thai CRVs

VP6



**Figure 8.3.** Phylogenetic tree of VP6. The maximum clade credibility (MCC) tree was constructed by BEAST 1.8 with the Bayesian Markov Chain Monte Carlo (BMCMC) algorithm. A strict clock model with a coalescent constant population and GTR, TN93 (G+I) and HKY with a gamma 4 substitution were used as model parameters. Pink circles indicate the Thai CRVs characterized in this study. Dog images (orange) represent the Thai CRVs and human images (black) represent the reference human RVs closely related to the Thai CRVs.

VP1

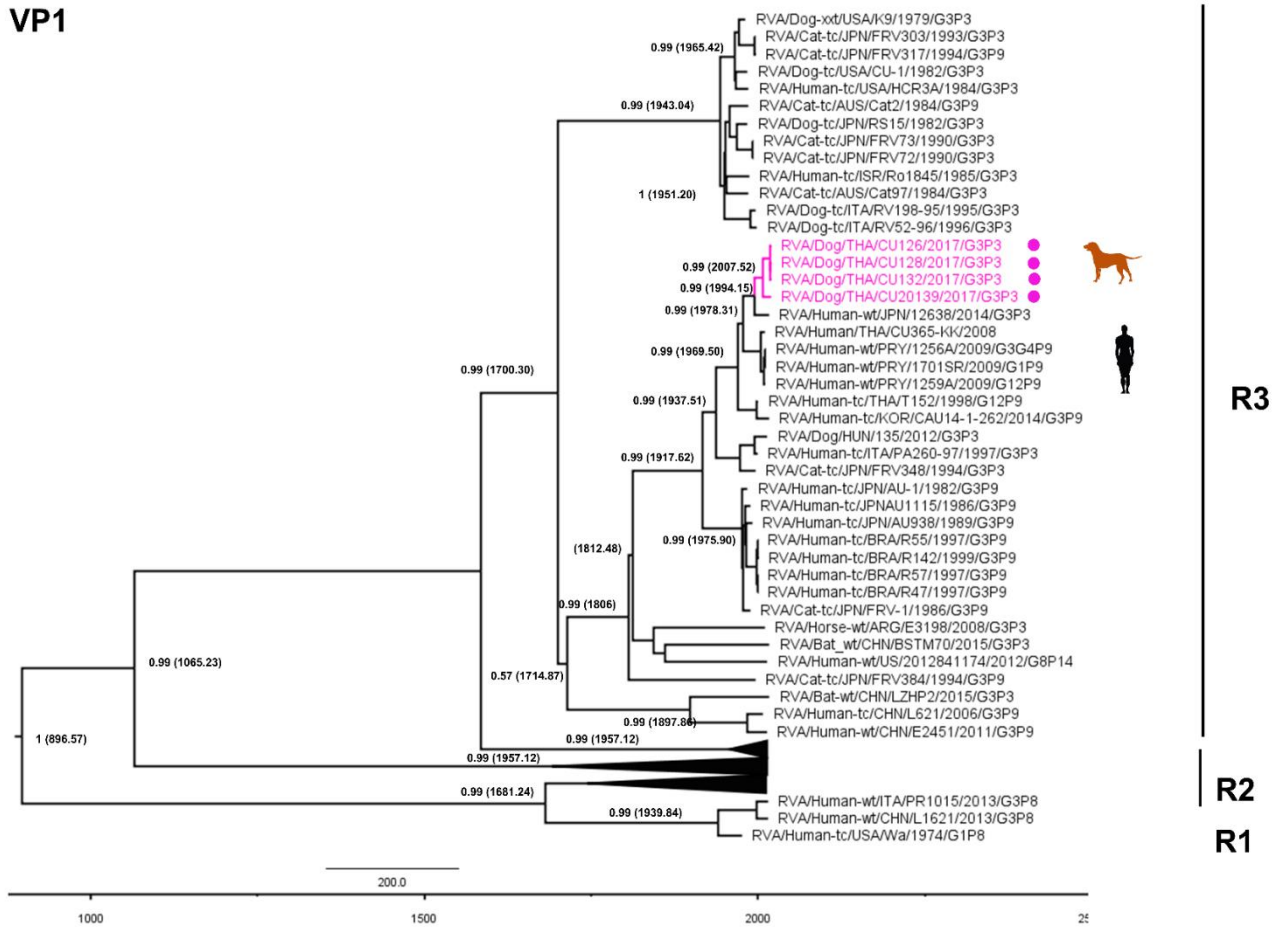
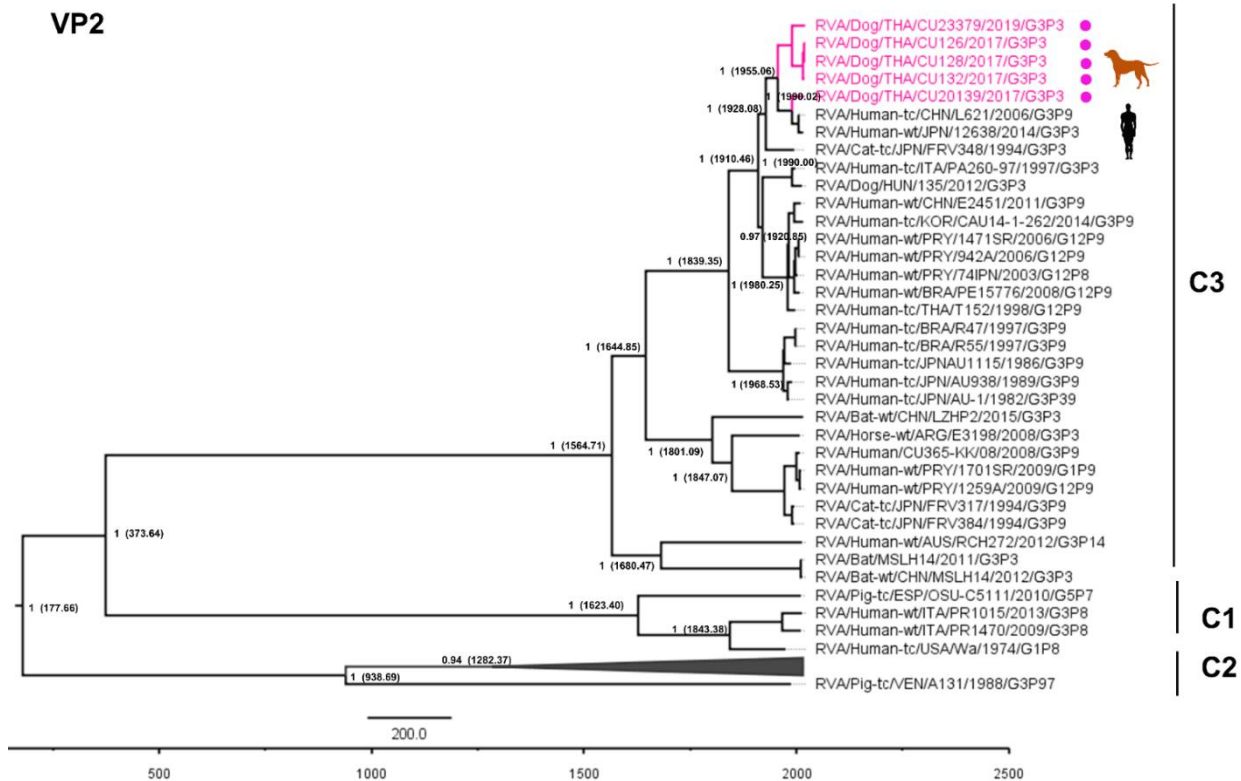


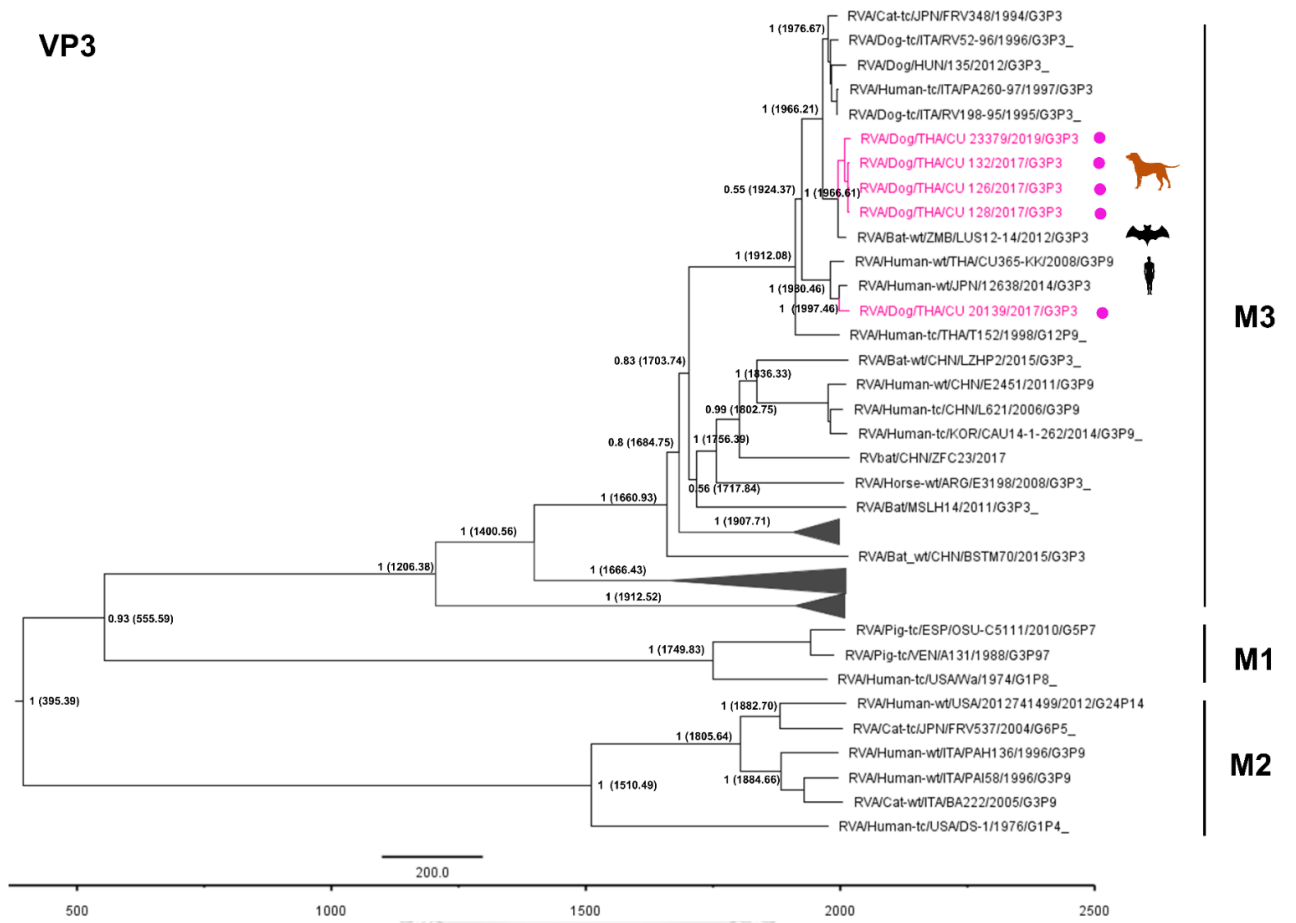
Figure 8.4. Phylogenetic tree of VP1 of Rotaviruses. Circles represent Thai CRVs characterized in this study.





**Figure 8.5.** Phylogenetic tree of VP2 of Rotaviruses. Circles represent Thai CRVs characterized in this study.





**Figure 8.6.** Phylogenetic tree of VP3 of Rotaviruses. Circles represent Thai CRVs characterized in this study.

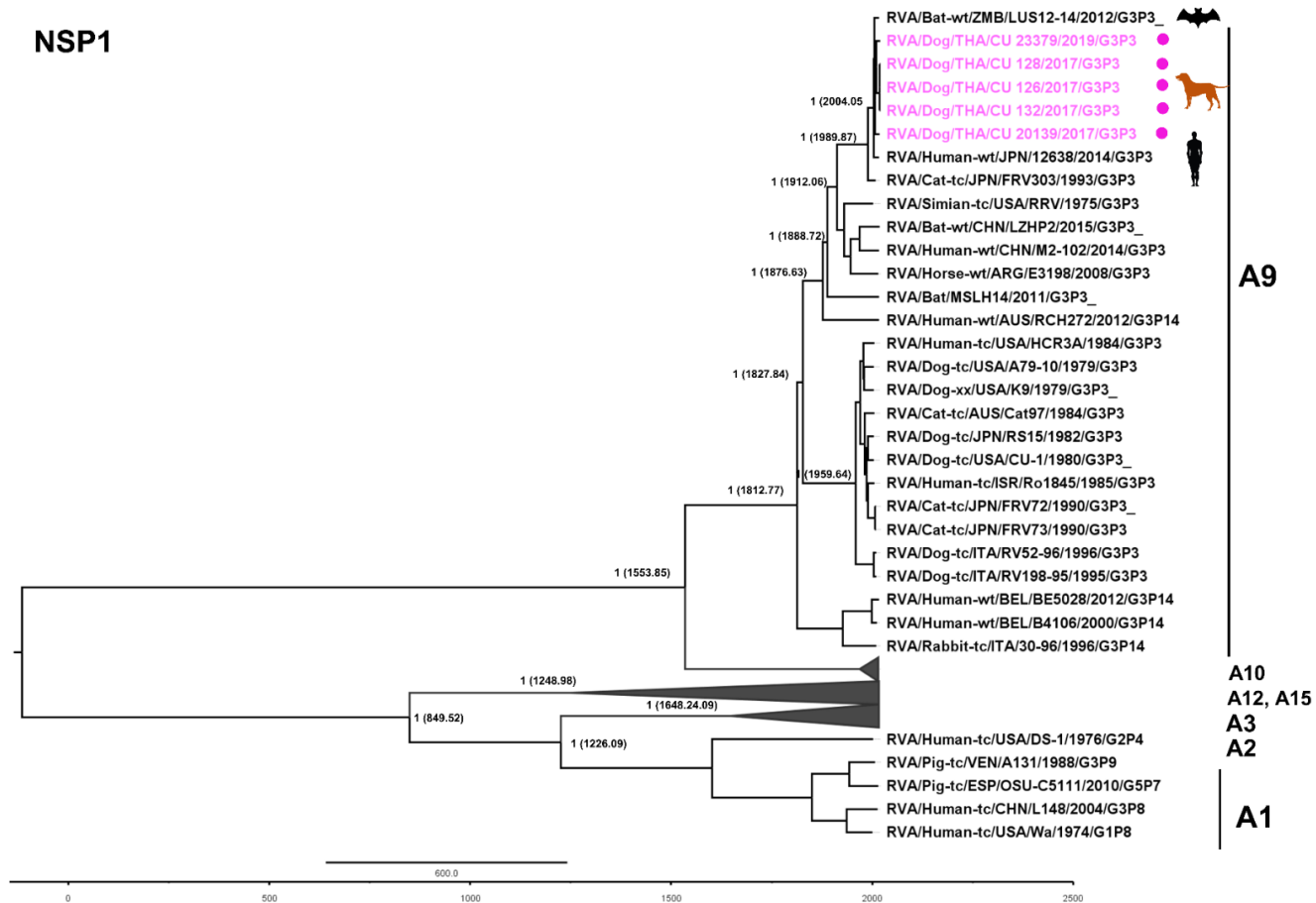
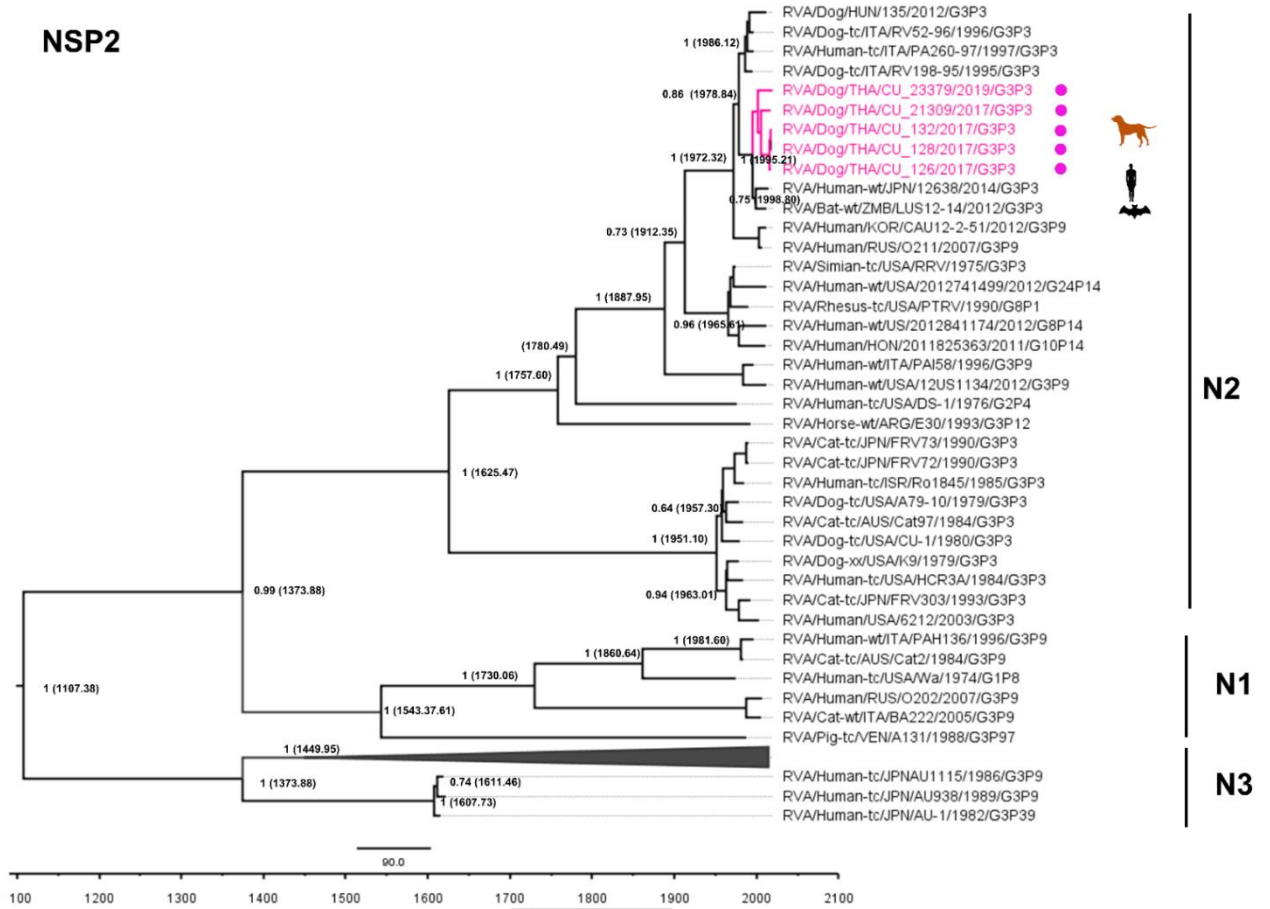
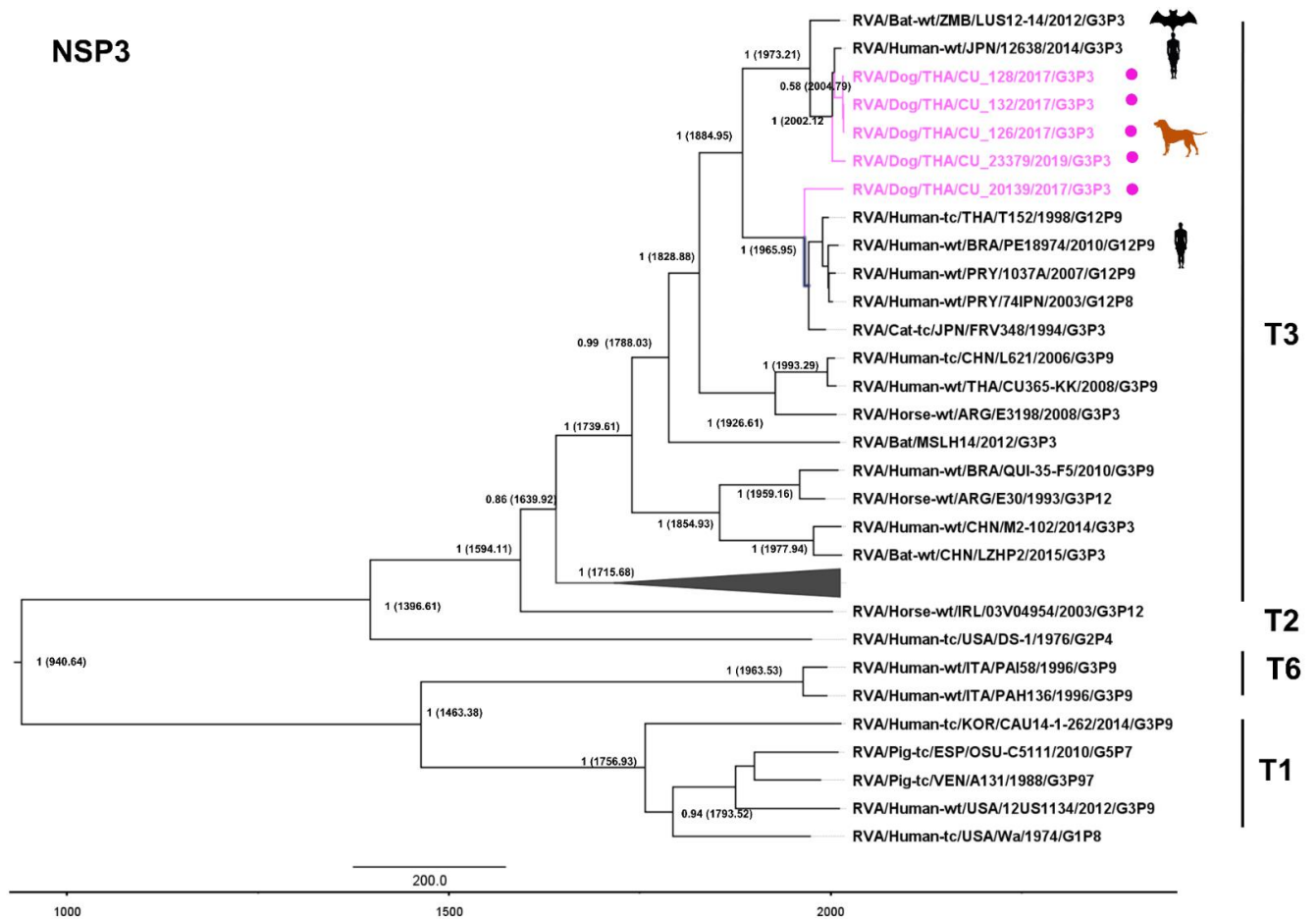


Figure 8.7. Phylogenetic tree of NSP1 of Rotaviruses. Circles represent Thai CRVs characterized in this study.

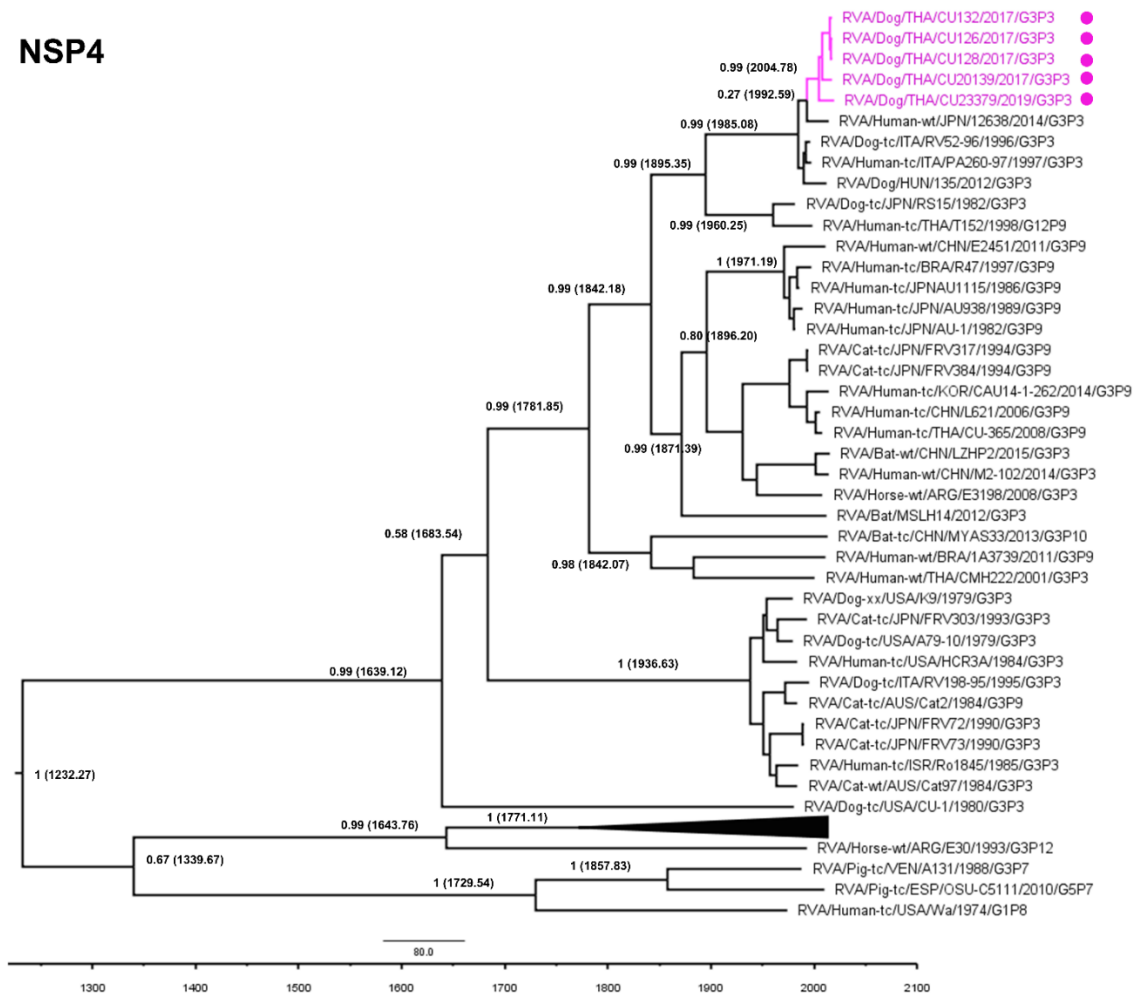


**Figure 8.8.** Phylogenetic tree of NSP2 of Rotaviruses. Circles represent Thai CRVs characterized in this study.



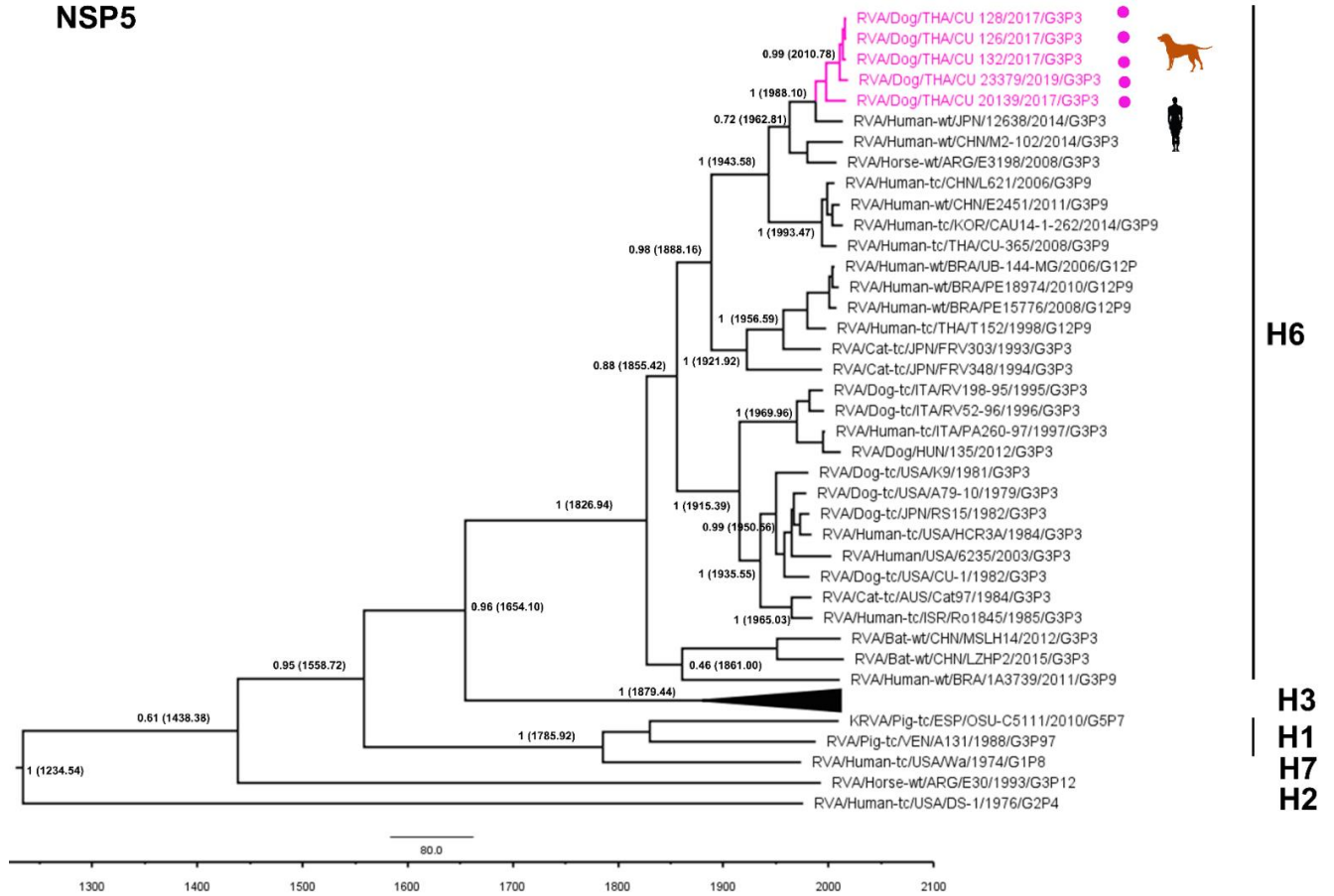
**Figure 8.9.** Phylogenetic tree of NSP3 of Rotaviruses. Circles represent Thai CRVs characterized in this study.

**NSP4**



**Figure 8.10.** Phylogenetic tree of NSP4 of Rotaviruses. Circles represent Thai CRVs characterized in this study.

**NSP5**



**Figure 8.11.** Phylogenetic tree of NSP5 of Rotaviruses. Circles represent Thai CRVs characterized in this study

#### 8.4.4. Genetic analysis of Thai canine rotaviruses

Genetic analyses of the nucleotide and deduced amino acids of the Thai CRVs were conducted by comparing the Thai CRVs against reference RVAs from humans, dogs, cats, bats and vaccine strains. The antigenic epitopes of the G3 genotype at regions 7-1a, 7-1b and 7-2, correlating with the blockade of neutralizing antibodies, were analyzed. The Thai CRVs contained amino acid substitutions in region 7-1b (A212V, N213S, K238D and D242T) and region 7-2 (T147A and A221T) which are also observed in reference rotaviruses from dogs and humans (HCR3A/USA, Ro1845/Israel, R2638/Brazil and 12638/Japan), but are different from the vaccine strain (Wi78-9 (RotaTeq™)) (Table 8.6). The Thai CRVs also had unique amino acids at positions 18F, 22M, 212V, and 221T which are only observed in reference viruses of the genotype G3, subcluster b2. The unique amino acids at positions 16F, 49K, 68A, 121E, and 238D were also found in the RVAs of cluster B (both subcluster b1 and b2), which can be used to differentiate cluster A and cluster B (Table 8.7).



**Table 8.6.** Genetic analysis of antigenic regions at VP7 among the Thai CRVs and reference RVAs from dogs, cats, bats, humans, and vaccine strain

Viruses	Country	Year	Amino acid position																												
			7-1a region							7-1b region							7-2 region														
			87	91	94	96	97	98	99	100	104	104	123	125	125	129	130	201	211	212	213	238	242	143	145	146	147	148	190	217	221
<b>Vaccine strain</b>																															
W178-9 (RotatEq™) (G3)**	USA	1992	T	T	N	N	S	W	K	D	Q	D	A	V	D	Q	D	A	N	K	D	K	D	A	T	L	S	E	A	G	
CU 20139	THA	2017	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	S	D	T	.	.	A	.	.	.	.	T	.	
CU 126	THA	2017	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	S	D	T	.	.	A	.	.	.	.	T	.	
CU 128	THA	2017	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	S	D	T	.	.	A	.	.	.	.	T	.	
CU 132	THA	2017	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	S	D	T	.	.	A	.	.	.	.	T	.	
CU23379	THA	2019	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	S	D	T	.	.	A	.	.	.	.	T	.	
<b>Canine</b>																															
K9	USA	1979	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	S	D	T	.	.	A	.	.	.	.	T	.	
A79-10	USA	1979	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	S	D	T	.	.	A	.	.	.	.	T	.	
<b>Feline</b>																															
Cat97	AUS	1984	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	S	D	T	.	.	A	.	.	.	.	T	.	
BA222	ITA	2005	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	N	N	.	.	.	.	.	.	.	.	.	
<b>Bat</b>																															
LUS12-14	ZMB	2012	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	A	D	T	.	.	N	.	.	.	.	.	.	
<b>Human</b>																															
AU-1	JPN	1982	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	N	N	.	.	.	.	.	.	.	.	.	
HCR3A	USA	1984	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	S	D	T	.	.	A	.	.	.	T	.	.	
Ro1845	ISR	1985	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	S	D	T	.	.	A	.	.	.	T	.	.	
1A3739	BRA	2011	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	T	T	D	A	.	.	N	.	.	.	.	.	.	
R2638	BRA	2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	S	D	T	.	.	A	.	.	.	T	.	.	
12638	JPN	2014	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	S	D	T	.	.	A	.	.	.	T	.	.	
CMH222	THA	2001	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	D	T	.	.	.	.	.	.	.	.	.	
CU365-HK	THA	2008	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	S	N	N	.	.	A	.	.	.	.	.	.	

\*Identical amino acid with vaccine strain (Rotarix-A41CA419A) are represented by dots.

\*\*Zeller et al., 2012

**Table 8.7.** Amino acid analysis of G3 between Thai CRVs and reference RVAs from dogs, cats, bats, humans representing cluster A, B and sub-cluster b1 and b2.

Viruses	Species	Country	Year	Lineage of G3	Amino acid position									
					Unique subcluster b2				Unique subcluster A					
					18	22	212	221	16	49	68	121	238	
<b>This study</b>														
CU 126	Canine	THA	2017	Lineage b2	F	M	V	T	F	K	A	E	D	
CU 128	Canine	THA	2017	Lineage b2	F	M	V	T	F	K	A	E	D	
CU 132	Canine	THA	2017	Lineage b2	F	M	V	T	F	K	A	E	D	
CU 20139	Canine	THA	2017	Lineage b2	F	M	V	T	F	K	A	E	D	
CU23379	Canine	THA	2019	Lineage b2	F	M	V	T	F	K	A	E	D	
<b>Reference RVA</b>														
K9	Canine	USA	1979	Lineage b2	F	M	V	T	F	K	A	E	D	
RV198-95	Canine	JPN	1995	Lineage b2	F	M	V	T	F	K	A	E	D	
A79-10	Canine	USA	1979	Lineage b2	F	M	V	T	F	K	A	E	D	
Cat97	Feline	AUS	1984	Lineage b2	F	M	V	T	F	K	A	E	D	
HCR3A	Human	USA	1984	Lineage b2	F	M	V	T	F	K	A	E	D	
Ro1845	Human	ISSR	1985	Lineage b2	F	M	V	T	F	K	A	E	D	
R2638	Human	BRA	2011	Lineage b2	F	M	V	T	F	K	A	E	D	
12638	Human	JPN	2014	Lineage b2	F	M	V	T	F	K	A	E	D	
HUN 135	Canine	HUN	2012	Lineage b1	L	I	T	A	F	K	A	E	D	
RV52-96	Canine	ITA	1996	Lineage b1	L	I	T	A	F	K	A	E	D	
Cat348	Feline	JPN	1994	Lineage b1	L	I	T	A	F	K	A	E	D	
LUS12-14	Bat	ZMB	2012	Lineage b1	L	I	T	A	F	K	A	E	D	
PA260-96	Human	ITA	1997	Lineage b1	L	I	T	A	F	K	A	E	D	
CMH222	Human	THA	2001	Lineage b1	L	I	T	A	F	K	A	E	D	
BA222	Feline	ITA	2005	Lineage A	L	V	T	A	V	N	T	D	N	
AU-1	Human	JPN	1982	Lineage A	L	V	T	A	V	N	T	D	N	
E2451	Human	CHN	2011	Lineage A	L	V	T	A	V	N	T	D	N	
CU365-KK	Human	THA	2008	Lineage A	L	V	T	A	V	N	T	D	N	

## 8.5 Discussion

To our knowledge, this study is the first to report on the genetics of canine rotavirus (CRV) in dogs in Thailand. Up to date, there are only seven complete genomes of canine rotavirus group A of genotype G3P[3] (Strain; RS15, RV198-95, VR52-96, A79-10, CU-1, K9 and HUN135) available in the GenBank database. Our results showed that the G3P[3] genotype was the predominant genotype circulating in dogs in Thailand, which is similar to other previous studies (Matthijnsens et al., 2011b; Papp et al., 2015; Tsugawa and Hoshino, 2008). From our survey, the samples collected in this study were from both healthy and symptomatic dogs of all breeds and ages from September 2016 to January 2019. Our results showed that only 0.70% (5/710) of the samples were positive for rotavirus A, which was lower than the prevalence in other studies (Alves et al., 2018; Mosallanejad et al., 2015; Ortega et al., 2017b). CRV could be detected in both sick and asymptomatic dogs similar to previous reports (Alves et al., 2018; Ortega et al., 2017b). CRVs were only detected in young dogs <1 year (Mosallanejad et al., 2015). It should be noted that the co-infection of CRV with canine parvovirus type 2 was observed in 2 out of 5 samples (data not shown).

Our study showed that Thai CRVs were varied from other reference CRVs, but more closely related to human RVAs. The Thai CRVs posed a novel genetic constellation “G3-P[3]-I3-R3-C3-M3-A9-N2-T3-E3-H6”, which has never been reported in CRVs from dogs but has been reported in RVAs from humans. Thai CRVs belong to genotype AU-1 with a combination of gene segments of genogroup Cat-like (10 segments) and AU-1-like (VP2). Based on our results, it can be speculated that Thai CRVs could have originated from multiple reassortment or intragenotype reassortment between the Cat97-like genogroup and the AU-1-like genogroup RVA. Similar findings have been reported in previous studies, for example human rotavirus strain 12638/Japan, PA260-97/Italy (De Grazia et al., 2007c; Okitsu et al., 2018).

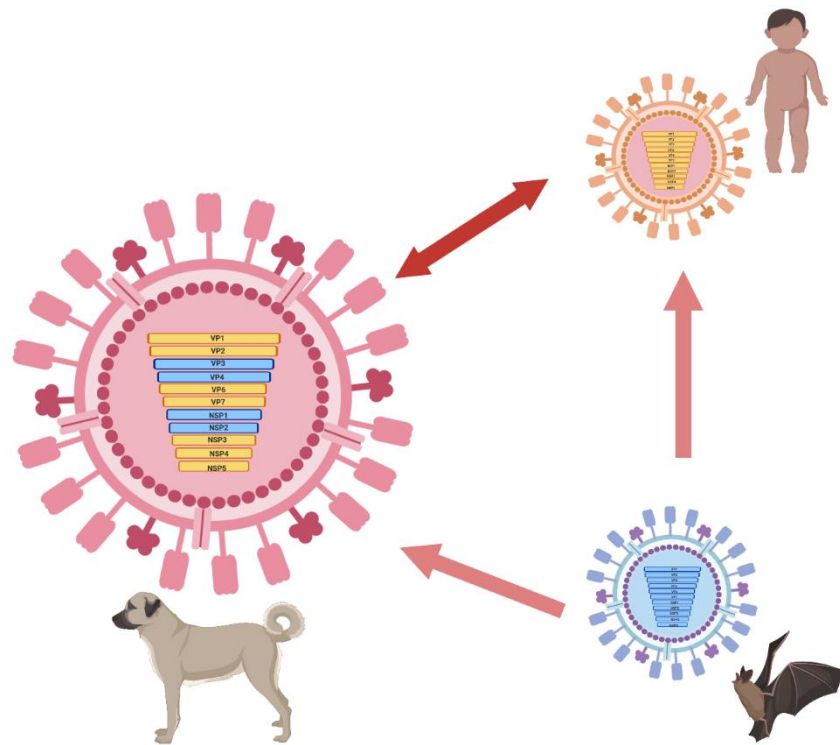
Based on phylogenetic analyses, our results showed that the Thai CRVs diverged from human RVA (12638/Japan) and bat RVA (LUS12-14/Zambia). The Thai

CRVs had high nucleotide identities with human RVA (12638/Japan), with 91.30-99.20% nucleotide identities (95.20-99.50% amino acid identities) and bat (LUS12-14) with 77.80-98.30% nucleotide identities (82.70-98.40% amino acid identities). The MCC analysis showed that VP1, VP2, VP3 (CU20139), VP6, VP7, NSP3, NSP4, and NSP5 of all Thai CRVs were estimated to separate from human RVA (12638/Japan) with the most recent common ancestor between 1965 and 2002. The VP3, VP4, NSP1, and NSP2 were estimated to separate from bat RVA (LUS12-14/Zambia) with tMRCA between 1966 and 1995. Our result suggested that Thai CRVs potentially originated from humans, bats and dogs through interspecies transmission that resulted in multiple reassortment of genes among humans and animals RVAs (Figure 8.12). However, a limitation of this study is the limited data of reference CRVs available in the GenBank database and only five positive samples were characterized in this study. Additionally, there are no information about contact history among human, bat and dog. Thus, this study does not provide conclusive evidence of cross-species transmission of CRV. Since our analysis was based on the available sequence data from the GenBank., if expansions of the nucleotide sequences of RVAs from various animal species becomes available, it would help us to better understand the interspecies transmission and multiple-reassortment of these viruses in the future.

In this study, the nucleotide substitution rates of all segments of the Thai CRVs are  $2.3015\text{-}5.3834 \times 10^{-4}$  (95%HPD:  $1.4649\text{-}8.0098 \times 10^{-4}$ ) (Table 8.4), which is similar to the estimated rate from other studies ( $9.7 \times 10^{-4}$ –  $4.1 \times 10^{-3}$ ) (Fujii et al., 2019; Jere et al., 2018). Moreover, our data indicated that the Thai CRVs had a higher genomic substitution rate than other dsRNA, approximately  $1 \times 10^{-5}$  substitutions per site per year (Firth et al., 2010). This result suggests the rapid evolution of the virus, and interspecies transmission of rotavirus is one of the factors activating this evolutionary mechanism. For example, the RVA genotype G3P[3] in humans has been reported worldwide such as in Brazil (R2638), Italy (PA260-97), USA (HCR3A), Israel (Ro1845), Japan (12638) and Thailand (CMH222), and the genetic constellation of

G3P[3] contains genes with an animal origin (De Grazia et al., 2007c; Khamrin et al., 2006b; Luchs et al., 2012; Okitsu et al., 2018; Tsugawa and Hoshino, 2008).

The genetic analysis result showed that the Thai CRVs posed characteristics of lineage b2 RVA. The analysis of antigenic epitopes of VP7 showed that the Thai CRVs posed a unique amino acid at region 7-1b (A212V, N213S, K238D and D242T) and region 7-2 (T147A and A221T) similar to canine and human RVAs (HUN/135, HCR3A/USA and 12638/Japan) but different from the commercial human rotavirus vaccine (Wi78-9 (RotaTeq™)). These unique amino acids are related to neutralizing antibodies and rotavirus vaccine efficacy (Dennehy, 2008; Zeller et al., 2012). The unique amino acids at these positions (16F, 49K, 68A, 121E, 238D) could be found in both subclusters b1 and b2, which can be used to differentiate clusters A and B. This observation has also been reported in a previous study in Japan (Okitsu et al., 2018). The Thai CRVs contained unique amino acids at positions 18F, 22M, 212V and 221T, suggesting unique amino acid determinants of lineage b2, which can be further used for diagnostic and genotyping purposes. However, the important and unique amino acids need further investigation.



**Figure 8.12.** Schematic presentation of possible multiple-reassortment of the Thai CRVs among the dog, bat, and human rotaviruses.

## 8.6 Conclusion

In summary, this study is the first to report canine rotaviruses genotype G3P[3] in dogs in Thailand. The Thai CRVs belonged to genotype AU-1 with gene segments of both genogroup Au-1-like and Cat 97-like, which have never been reported before in any canine rotaviruses. Phylogenetic analyses showed that the Thai CRVs were closely related and might have originated from human and bat RVAs with multiple-reassortment. Thus, dog owners and veterinary practitioners should pay more attention to rotavirus infection as a potentially zoonotic and reverse zoonotic viral disease. There is a need to survey CRVs on a larger scale to determine the dynamics and distribution of rotaviruses in Thailand.



## CHAPTER IX

### Surveillance of potential respiratory and enteric zoonotic viruses in high-risk occupations in Bangkok, Thailand

The manuscript is in preparation in title

#### Human coronavirus in persons with high-risk occupations, Thailand.

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#### 9.1. Abstract

Dog-human interface is common in urban settings which could be one of the factors to elevate the risk of zoonotic diseases from dogs to humans, especially among persons who have routinely close contact with dogs. This cross-sectional study aimed to determine the distribution of the coronavirus infection among persons who have been closed contact with dogs (e.g., veterinarians, animal care workers and veterinarian's assistants) from small animal hospitals in Bangkok during June 2018 to May 2019. The nasal swab samples (n=100) and stool samples (n=100) were collected from participants for viral identification. The nasal swab samples were tested for influenza virus (IAV), parainfluenza virus (PIV) and coronavirus (CoV) while stool samples were tested for coronavirus (CoV) and rotavirus (RV). Our results showed that two participants were positive for CoV, while none of the other viruses (IAV, PIV and RV) were detected. The genetic characterization of 2 positive CoVs showed that the viruses were clustered with human alphacoronavirus (HCoV- 229E). The results from the questionnaire analysis showed that 52% of participants reported they have risk of zoonotic infection from dogs. Moreover, 93% of participants use appropriate PPE when in contact with dogs. In conclusion, this study provided the



information of potential zoonotic viruses, genetic characteristics, and information on knowledge and practices that might impact zoonotic infection in dogs and humans. However, this study conducted and analyzed a relatively small population. Therefore, further study research should be implemented on larger scale for better understanding the dynamics and distributions of potential zoonotic diseases in dogs and humans in Thailand.



## 9.2. Introduction

CoV is an enveloped, non-segmented, single-stranded RNA virus. CoV belongs to the family *Coronaviridae* which can be classified into four genus including alphacoronavirus, betacoronavirus, deltacoronavirus and gammacoronaviruses. The virus contains structural proteins including spike protein (S), membrane protein (M), envelop protein (E), haemagglutinin esterase protein (HE) and nucleocapsid protein (N) (Woo et al., 2010a). CoVs cause systemic infection (respiratory, enteric and neurological symptoms) in several animal species as well as human CoVs poses a threat to human because the viruses have high mutation and evolutionary rate which can generate novel or virulence viruses for example SARS-CoV, SARS-CoV-2, MERS (Chafekar and Fielding, 2018; Holmes and Rambaut, 2004; Rajendran et al., 2020).

There are 4 stains of common respiratory coronavirus in human (HCoV) including HCoV- 229E, HCoV- OC43, HCoV- NL63 and HCoV- HKU. HCoVs can infect upper respiratory tract and cause mild clinical signs and self-limiting (Liu et al., 2020). Moreover, MERS-CoV and SARS-CoV had been reported to infect human and cause severe respiratory signs in 2003 and 2012, respectively (Chafekar and Fielding, 2018; Holmes and Rambaut, 2004). In 2019-2020, the novel coronavirus (SARS-CoV-2) was first reported in China (Zhu et al., 2020). This SARS-CoV-2 causes COVID-19 and severe respiratory disease and spread worldwide causing pandemic outbreaks. The origin of this novel virus (SARS-CoV-2) has been speculated from cross species transmission from animals (bats, pangolin) to human. Several studies reported that the novel SARS-CoV-2 virus spilled over from human to animals (dogs, cats, tigers, lions and mink) (Halfmann et al., 2020; McAloose et al., 2020; Munnink et al., 2021; Sit et al., 2020a). Especially, in domestic animals with a history of close contact with COVID-19 patients (Patterson et al., 2020).

Canine respiratory virus (CRCoV) is CoV circulating and causing respiratory signs in dogs. The genetic of CRCoV is closely related to human coronavirus (HCoV-OC43) and bovine coronavirus (BCoV) (Erles et al., 2007). Previous study suggested that CRCoV, HCoV-OC43 and BCoV have common ancestor and have ability to cross-species transmission (Vijgen et al., 2005). In Thailand, human CoVs and bovine CoVs have been reported (Singasa et al., 2017; Soonnarong et al., 2016).

Since dog-human interface is common and is considered as one of the factors to elevate the risk of zoonotic diseases transmission from dogs to humans, especially among persons who have routinely close contact with dogs such as veterinarian and animal care workers (Baker and Gray, 2009). In Thailand, the information of respiratory coronaviruses transmission between dogs and humans who have high risk occupations are still less numbers. Therefore, this study aimed to conduct a survey of respiratory coronaviruses in persons with high-risk occupations in Bangkok during June 2018 to May 2019.



### 9.3. Materials and Methods

#### 9.3.1 Human sample collection

This analytic cross-sectional study was conducted to determine the status of and distribution of coronaviruses among persons who have been in closed contact with dogs (e.g., veterinarians, animal care workers and veterinarian's assistants) from 12 small animal hospitals in Bangkok during June 2018 to May 2019. Inclusion criteria for small animal hospital selection were 1) large scale animal hospitals with more than 10 in-patient admissions, 2) animal hospitals with routine treatment of canine respiratory and enteric diseases and 3) cooperation of animal hospital owners and veterinarians. The inclusion criteria for participants were 1) persons who have a high risk of exposure to dogs such as veterinarians and animal care workers, 2) persons who have been working in animal hospitals for more than a month and 3) the cooperation of participants in sample collection. Approximately 100 persons participated in the study, face-to-face interviews were used to gather information by using a questionnaire. The participants were also asked to collect nasal swab and stool samples for virus detection. The sampling procedures were conducted following the U.S. Department of Health and Human Services, Centers for Disease Control and Prevention (CDC)'s recommendation available at [www.cdc.gov/urdo](http://www.cdc.gov/urdo). This study was conducted under the approval protocol of the Chulalongkorn University Institutional Review Boards (IRBs) (No.# 085.1/61).

#### 9.3.2. Questionnaire

Questionnaire data was used to identify factors of high-risk occupations on the exposure with potential zoonotic respiratory and enteric viruses such as occupation, frequency of exposure to sick dogs, personal protective equipment (PPE) when handling with sick dogs, personal hygiene when handling with sick dogs (handwashing) and the use of detergents or disinfectants when exposed to sick dogs.

### 9.3.3. Human coronavirus identification

Human samples were processed at the laboratory of the center of excellence for Clinical Virology, Faculty of Medicine, Chulalongkorn University. Stool samples were processed according to the WHO recommendation. In brief, 10-fold dilution of stool sample in phosphate-buffered saline (PBS) were aliquoted and processed for DNA and/or RNA extraction (Kittigul et al., 2014). Nasal swab and dilution of rectal samples were processed for RNA extraction by using the QIAmp viral RNA mini kit (Qiagen, Hilden, Germany) following manufacturer's suggestion. RNA was stored at –20°C until use. All nasal swab (n=100) and stool (n=100) samples were identified for CoV by using one-step RT-PCR with RdRp gene specific primers. The primers used in this study were previously described (Lelli et al., 2013). Briefly, one-step RT-PCR (Invitrogen, USA) was used in a total final volume of 30 µl comprising 3 µl of template RNA, 15 µl of 2xReaction Mix, 0.6 µl of 10 µM forward (5'-GGTTGGGACTATCCTAAGTGTGA-3') and reverse primer (5'-CCATCATCAGATAGAATCATCATA-3'), 1.2 µl of SuperScript III RT and distilled water to final volume 30 µl. For RT-PCR condition, the reaction contained cDNA synthesis step at 55°C for 30 minutes, next to an initial denaturation step at 94 °C for 2 min, following 40 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 30 s and extension at 68 °C for 30 s, final extension step at 68 °C for 6 min. To confirm CoV, 4 µl of PCR product was run on a 1.5% agarose gel electrophoresis. The expected amplified product size of CoV was 440 bp. Other respiratory viruses and enteric viruses were also tested (human parainfluenza virus, influenza A, B virus, rotavirus group A) but all of them were negative.

### 9.3.4. Coronavirus characterization

In this study, HCoV (n=2) were characterized by partial RdRp sequencing with primer specific to RdRp gene. The nucleotide sequencing was conducted at the 1<sup>st</sup> Base Laboratories Sdn Bhd, Malaysia. The nucleotide sequences were validated and assembled by SeqMan software v.5 v.5.03 (DNASTAR Inc.; Wisconsin, USA). The phylogenetic analysis was conducted by comparing nucleotide sequences of Thai CoV with those of CoV available from the GenBank database. The reference

nucleotide sequences of CoVs were retrieved based on their different geographic locations, host species. Phylogenetic analysis was performed by using MEGA v.6.0 (Tempe, AZ, USA) applying neighbor-joining method with Kimura 2-parameter with 1,000 bootstrap replicates (Tamura et al., 2013). For genetic analysis, the nucleotide sequences and deduced amino acids of HCoV were aligned and compared by using MegAlign software v.5.03 (DNASTAR Inc.; Wisconsin, USA).

#### 9.4. Results

In this study, we investigated coronavirus infection in humans with high-risk occupations (veterinarians, animal care workers and veterinarian's assistants) from small animal hospitals in Bangkok, Thailand. We detected 2% (2/100) of CoV from human nasal swab samples. However, CoV could not be detected from stool samples (0/100). In this study, there were no positivity for other respiratory and enteric viruses including influenza A, B and paramyxovirus and rotavirus (data not showed). The partial RdRp gene sequences of Thai HCoV (CU-H1 and CU-H2) were elucidated (Table 9.1). Analysis of nucleotide identities of RdRp gene showed that Thai HCoVs (CU-H1) possessed highest nucleotide identities to HCoV-229E from Thailand (CU-A54) (100% nucleotide identities and amino acid identities) (Table 9.2). Phylogenetic analysis of RdRp gene showed that Thai-HCoV (CU-H1 and CU-H2) were clustered with the HCoV-229E of the alphacoronavirus and closely related to HCoV-229E from Thailand (Figure 1). It is noted that the nucleotide identities and phylogenetic tree revealed that Thai HCoVs from this study were clustered into separate group from Thai CRCoVs with 56.80-57.10% nucleotide identities and 51.90% amino acid identities (Table 9.2).

**Table 9.1.** Detail description of human coronaviruses (HCoV) characterized this study.

Virus ID	Date of isolation	Breed	Sequencing	GenBank accession number
<b>HCoV</b>				
CU-H1	Jan-19	Human	RdRp	Processing
CU-H2	Feb-19	Human	RdRp	Processing



**Table 9.2.** Pairwise comparison of nucleotide sequences of RdRp gene of Thai HCoV and reference coronaviruses.

Virus	Accession No	Host	Country	Year	RdRp gene CU-H1	
					% Nucleotide identities	% Amino acid identities
<b>HCoV this study</b>						
CU-H1		Human	Thailand	2019	100.00	100.00
CU-H2		Human	Thailand	2019	100.00	100.00
<b>CRCoV this study</b>						
AD21		Dog	Thailand	2016	56.80	51.90
AD 431		Dog	Thailand	2017	57.10	51.90
<b>Reference strains</b>						
<b>CRCoV</b>						
BJ232	KX432213	Dog	china	2014	56.80	51.90
K37	JX860640	Dog	South Korea	2008	57.70	52.80
<b>BCoV</b>						
Oh-440-tc	NC 012949	Bovine	USA	1996	58.30	52.80
DB2	DQ811784	Bovine	USA	1983	58.00	52.80
AH187	NC 012948	Bovine	USA	2000	58.30	52.80
Kakegawa	AB354579	Bovine	Japan	1980	57.70	52.80
<b>HCoV-OC43</b>						
CU-C437	KJ866100	Human	Thailand	2012	59.60	53.70
901-43	KF530061	Human	USA	1990	59.60	53.70
ATCC VR-759	AY391777	Human	USA	1967	59.60	53.70
<b>HECoV-4408</b>						
HECoV/4408/	FJ415324	Human	Germany	1988	58.30	52.80
HECoV/4408	NC 012950	Human	USA	2009	58.30	52.80
<b>SARS-CoV</b>						
Tor2	NC 004718	Human	China	2003	55.90	55.60
<b>SARS-CoV2</b>						
NY/040420	MT365033	Tiger	USA	2020	57.10	55.60
Wuhan-Hu-1		Human	China	2019	57.10	55.60
20-03695	MT270814	Dog	Hong Kong	2020	57.10	55.60



**Table 9.2.** Pairwise comparison of nucleotide sequences of RdRp gene of Thai HCoV and reference coronaviruses (cont.)

Virus	Accession No	Host	Country	Year	RdRp gene CU-H1	
					% Nucleotide identities	% Amino acid identities
<b>MERS</b>						
HCoV-EMC	JX869059	Human	Netherlands	2012	58.00	54.60
<b>CoV</b>						
1-71	JQ404409	Dog	Germany	1971	70.10	75.90
23/03	KP849472	Dog	Italy	2003	70.40	75.00
CB/05	KP981644	Dog	Italy	2006	69.40	75.90
<b>FCoV</b>						
FIP 79-1146	NC 002306	cat	USA	1979	70.70	75.90
79-1683	JN634064	cat	USA	1970	69.40	75.00
<b>HCoV-NL63</b>						
CU-C2735/	KJ866087	Human	Thailand	2012	74.70	80.60
HCoV/NL63	AY518894	Human	Netherlands	1988	74.40	79.60
<b>HCoV-229E</b>						
HCoV/229E	AF304460	Human	UK	1963	99.40	100.00
933-40	KF514433	Human	USA	1993	99.70	100.00
CU-B8003	KJ866057	Human	Thailand	2013	98.80	98.10
CU-A54	KJ866056	Human	Thailand	2013	100.00	100.00
<b>PED</b>						
CV777	NC 003436	Pig	Belgium	1977	69.40	75.00
<b>TGEV</b>						
Purdue	AJ271965	Pig	Spain	2000	68.20	75.00

In this study, we also performed questionnaire interviews from 100 participants who have high risk occupations. Our results showed that the demographic characteristics of the participants were mostly between 20-39 years old (85%), majority female (73%) and 60% of participants held bachelor's degrees. Of the participants, 63% self-identified as animal care workers, and 37% identified as veterinarians. Nearly all participants (95%) reported working more than 48 hours per week, and 20% reported exposure to more than 10 sick dogs per week. 52% of workers reported that they have risk of zoonotic infection from dogs. Of the 16% participants who had previously been diagnosed with zoonotic infection from doctors with 87.5% were a fungal infection (Table 9.3). For zoonotic awareness in the hospitals, more than 80% of the clinical workers reported that their workplace provided PPE instruments (e.g., detergent, gloves, mask, and coat) and separated areas between animals and humans (Table 9.4). For the practice of people who had closely contact with sick dog, nearly half of the workers (45%) when exposed to sick dogs conducted proper personal hygiene and used PPE (e.g., hand washing, equipment disinfect, coat changing, avoid eating during work). However, the 8% of participants reported seldom using of gloves when working with sick dogs and 37 % of participants reported seldom use of masks when exposed to dogs with respiratory illnesses (Table 9.5-9.6). For knowledge attitude to zoonotic infection from dog, more than 80% of participant were well known that pathogens causing zoonotic disease such as dermatophytosis, leptospirosis, and Rabies. While only 36 % of participants were know that influenza, coronavirus and rotavirus are causing zoonosis and reverse zoonosis from humans to animals. Most (84%) had the knowledge of possible causes and prevention of zoonotic infections and 63% of participants were interested in participating in training on zoonotic diseases (Table 9.7).

**Table 9.3.** Demographic characteristics of high-risk occupations who have been in close contacted with sick dogs (n=100)

Demographics		number	%
1	<b>Age group</b>		
	20-39	85	85
	40-59	14	14
	>60	1	1
2.	<b>Gender</b>		
	Male	27	27
	Female	73	73
3.	<b>Education attainment</b>		
	Primary School	11	11
	Junior High School	6	6
	Senior High School	15	15
	Bachelor's degrees	60	60
	Master's degrees	8	8
4	<b>Occupation</b>		
	Veterinarian	37	37
	Veterinarian Assistance	73	73
5	<b>Working hour / week</b>		
	More than 48 hour /week	95	95
	Less than 48 hour/week	5	5
6	<b>A month period, how many expose dogs with respiratory signs</b>		
	No	1	1
	1-3 ill dogs / week	35	35
	4-6 ill dogs /week	27	27
	7-9 ill dogs /week	17	17
	>10 ill dogs /week	20	20
7	<b>A month period, how many expose dog with gastroenteritis signs</b>		
	No	3	3
	1-3 ill dogs / week	34	34
	4-6 ill dogs /week	30	30
	7-9 ill dogs /week	16	16
	>10 ill dogs /week	17	17
8	<b>Had ever been diagnosed with zoonotic infection from doctor</b>		
	no	84	84
	yes	16	16

**Table 9.4** Information of small animal hospital management regarding to zoonotic diseases.

Small animal hospital Management		n	%
<b>1</b>	<b>Separated areas between healthy and sick animals</b>		
	Yes	82	82
	No	17	17
	No answer	0	0
<b>2</b>	<b>separated areas between animals and humans</b>		
	Yes	87	87
	No	12	12
	No answer	1	1
<b>3</b>	<b>Provided PPE instruments when working /exposure with sick animal shoes</b>		
	Yes	62	62
	No	38	38
	No answer	0	0
<b>4</b>	<b>Coat</b>		
	Yes	74	74
	No	26	26
	No answer	0	0
<b>5</b>	<b>Disposable glove</b>		
	Yes	98	98
	No	2	2
	No answer	0	0
<b>6</b>	<b>Mask</b>		
	Yes	92	92
	No	8	8
	No answer	0	0
<b>7</b>	<b>Goggle</b>		
	Yes	89	89
	No	9	9
	No answer	2	2

**Table 9.4.** Information of small animal hospital management regarding to zoonotic diseases. (cont.)

Small animal hospital Management		n	%
<b>8</b>	<b>Equipment and hand washing facilities</b>		
	Yes	100	100
	No	0	0
	No answer	0	0
<b>9</b>	<b>Detergent/disinfectant</b>		
	Yes	100	100
	No	0	0
	No answer	0	0



**Table 9.5.** Practices of persons who have been in close contact with dogs with gastroenteritis signs.

	Practice /procedure	(n)	%
1	<b>Used specific shoes in working area</b>		
	Never	30	30
	Rarely	4	4
	Sometimes	21	21
	Usually	13	13
	always	32	32
2	<b>Coat</b>		
	Never	22	22
	Rarely	4	4
	Sometimes	14	14
	Usually	13	13
	always	47	47
3	<b>Disposable glove</b>		
	Never	2	2
	Rarely	5	5
	Sometimes	23	23
	Usually	18	18
	always	52	52
4	<b>Protective mask</b>		
	Never	15	15
	Rarely	21	21
	Sometimes	31	31
	usually	17	17
	always	16	16
5	<b>Hands washing with soap/disinfectant before expose dog</b>		
	Never	16	16
	Rarely	9	9
	Sometimes	11	11
	usually	18	18
	always	46	46
6	<b>Hands washing with soap/disinfectant After expose dog</b>		
	Never		
	Rarely	1	1
	Sometimes	6	6
	usually	17	17
	always	76	76

**Table 9.5.** Practices of persons who have been in close contact with dogs with gastroenteritis signs. (cont.)

	Practice /procedure	(n)	%
7	<b>Changing coat/equipment before exposed next other dogs</b>		
	Never	37	37
	Rarely	13	13
	Sometimes	23	23
	usually	11	11
	always	16	16
8	<b>Drinking /eating food when working</b>		
	Never	55	55
	Rarely	9	9
	Sometimes	30	30
	usually	4	4
	always	2	2
9	<b>Drinking /eating food after exposed sick dog</b>		
	Never	29	29
	Rarely	12	12
	Sometimes	28	28
	usually	12	12
	always	9	9
10	<b>equipment disinfectant</b>		
	Never	1	1
	Rarely	0	0
	Sometimes	4	4
	usually	12	12
	always	83	83

**Table 9.6.** Practices of persons who have been in close contact with dogs with respiratory signs.

	Practice /procedure	(n)	%
1	<b>Use specific shoes in working area</b>		
	Never	30	30
	Rarely	3	3
	Sometimes	18	18
	Usually	17	17
	always	32	32
2	<b>Coat</b>		
	Never	24	24
	Rarely	1	1
	Sometimes	10	10
	Usually	15	15
	always	50	50
3	<b>Disposable glove</b>		
	Never	4	4
	Rarely	4	4
	Sometimes	25	25
	Usually	18	18
	always	49	49
4	<b>Protective mask</b>		
	Never	20	20
	Rarely	17	17
	Sometimes	32	32
	usually	13	13
	always	18	18
5	<b>Hands washing with soap/disinfectant before expose dog</b>		
	Never	14	14
	Rarely	5	5
	Sometimes	18	18
	usually	17	17
	always	46	46
6	<b>Hands washing with soap/disinfectant After expose dog</b>		
	Never	3	3
	Rarely	1	1
	Sometimes	10	10
	usually	14	14
	always	72	72

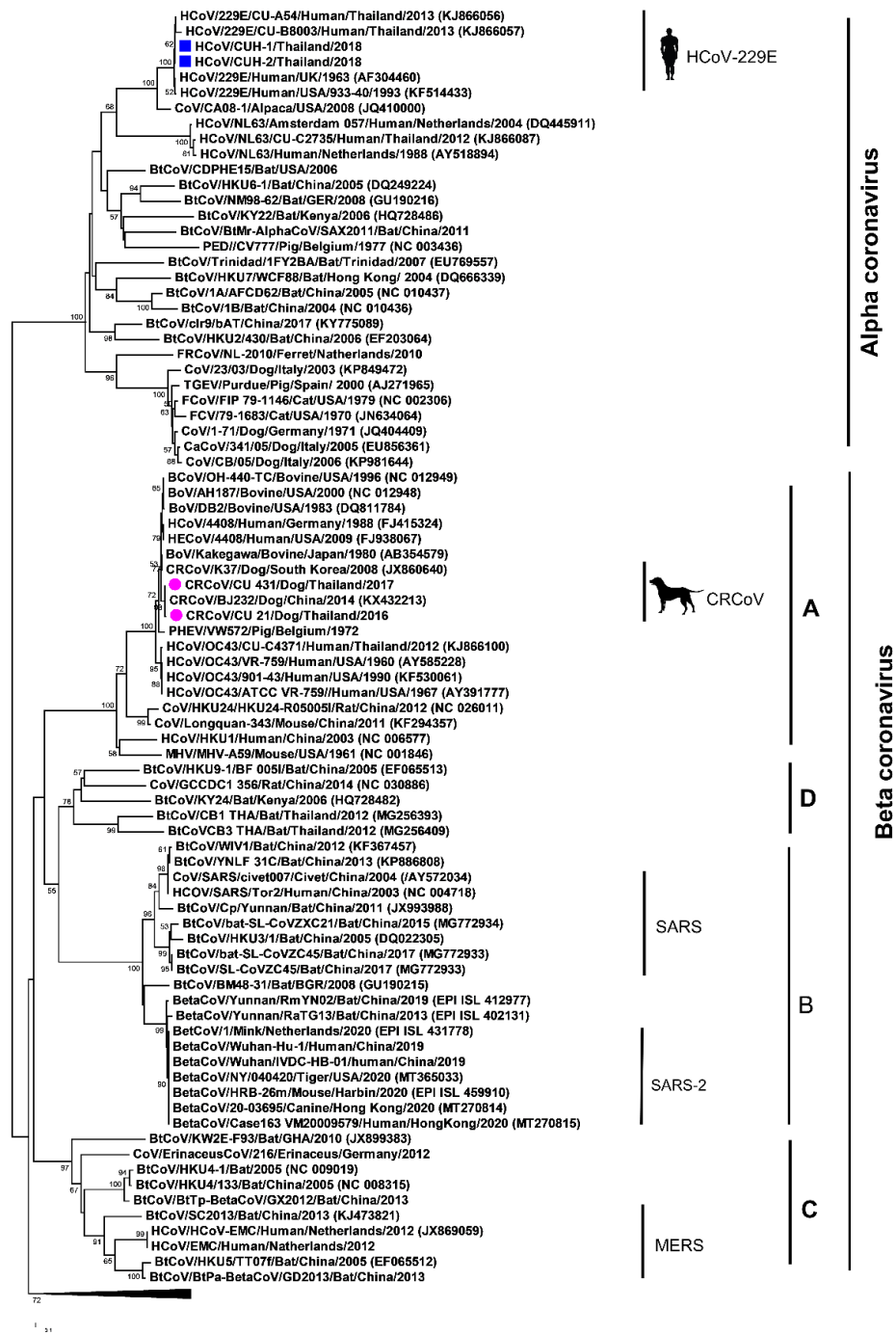


**Table 9.6.** Practices of persons who have been in close contact with dogs with respiratory signs. (cont.)

Practice /procedure	(n)	%
<b>7 Changing coat/equipment before exposed next other dogs</b>		
Never	36	36
Rarely	16	16
Sometimes	27	27
usually	8	8
always	13	13
<b>8 Drinking /eating food when working</b>		
Never	53	53
Rarely	11	11
Sometimes	27	27
usually	8	8
always	13	13
<b>9 Drinking /eating food after exposed sick dog</b>		
Never	29	29
Rarely	15	15
Sometimes	35	35
usually	14	14
always	7	7
<b>10 equipment disinfectant</b>		
Never	1	1
Rarely	1	1
Sometimes	4	4
usually	7	7
always	87	87

**Table 9.7.** Summary of knowledge, attitudes, and experience of participants in this study

Knowledge, attitudes, and experience		(n)	%
<b>1</b>	<b>Do you have risk to infect with zoonotic disease from dog</b>		
	No	52	52
	Yes	30	30
	Not sure	17	17
<b>2</b>	<b>Which pathogens casing zoonotic pathogen</b>		
	Dermatophyte	85	85
	Rabies	83	83
	Leptospirosis	77	77
	Parvovirus	8	8
	Coronavirus	13	13
	Rotavirus	27	27
	Influenza virus	36	36
	Parainfluenza virus	17	17
	Not answer	10	10
<b>3</b>	<b>Route of transmission</b>		
	Direct contact	73	73
	Consuming	56	56
	biting	95	95
	Breathing	51	51
	blood	44	44
<b>4</b>	<b>Which topic of zoonotic disease do you would like to have more Information</b>		
	Do not want to know	2	2
	Zoonotic pathogens	84	84
	Pathogenesis	65	65
	Clinical sign and severity	80	80
	Protection	88	88
<b>5</b>	<b>It this is training educate about zoonotic disease</b>		
	Participate	63	63
	No	25	25
	Not sure	12	12



**Figure 9.1.** Phylogenetic tree of Thai HCoV based on RdRp gene. The phylogenetic tree was constructed by using MEGA v.7.0 with neighbor-joining algorithm with kimura-2 parameter model with bootstrap analysis of 1,000 replications. Blue squares presented HCoV in this study. Pink circulars presented canine CoV (CRCoV) in this study.

## 9.5. Discussion

Our results demonstrated that HCoVs were circulating in persons with high-risk occupations (veterinarian, veterinarian's assistants, and animal care workers). The analysis of nucleotide sequence of RdRp gene of HCoVs showed that Thai HCoVs (CU-H1 and CU-H2) were grouped with HCoV-299E of Alphacoronavirus. The RdRp gene of two Thai-HCoVs in this study had highest nucleotide identities to HCoV-299E strain CU-A54 from Thailand (100% nucleotide identities and amino acid identities). Thai HCoVs from this study were clustered in separated group from Thai CRCoVs with 56.80-57.10% nucleotide indicating no relation between human CoV (HCoV-229E) and canine CoV (CRCoV) characterized in this study. However, coronavirus has high mutation rate that can generate novel virus and has ability to cross-species transmission. Recently, the SARS-CoV-2 have been reported to cross-species transmission from humans to dogs. Our result showed that Thai canine respiratory coronavirus (CRCoV) was grouped betacoronavirus group A which closely related with HCoV-OC43 and BCoV.

Our survey results demonstrated that most (80%) of small animal hospitals provide a safe workplace for their staffs such as separate area between animals and humans, provide PPE (disposable glove, coat, mask, detergent, disinfectant and hand washing facility). The personal hygiene (washing hand, avoid eating, or drinking when animal handling) and using PPE when handling sick animal were conducted by participants in this study and our results showed that higher proportion that previous studies (36-48%) (Baker and Gray, 2009; Lipton et al., 2008). For knowledge attitude to zoonotic infection from dogs, more than 80% of participants were well known that common pathogens causing zoonotic disease such as dermatophytosis, leptospirosis, and Rabies. While other potential zoonosis and reverse zoonosis (influenza, coronavirus and rotavirus are causing) might need more education regarding to the prevention and control. Moreover, personal hygiene (hand washing, avoid eating during animal handling) and using PPE (gloves, mask, and coat) need to improve with goal of 100% following the National Association of State Public Health Veterinarians (NASPHV) (Williams et al., 2015). Our recommendations to the small animal hospitals were to develop and implement personal hygiene trainings and infection-control

guidelines.

### 9.6. Conclusion

In conclusion, this study provided information of potential zoonotic viruses, genetic characteristic, and information on knowledge and practices that might impact zoonotic infection in humans and dogs in Bangkok, Thailand. This information can be used by small animal hospital owners and practitioners (veterinarians and animal care workers and veterinarian's assistants) to develop risk communication guidance for high-risk occupations. However, this study analyzed a relatively small population and did not obtain many positive samples, thus it is not conclusive. Therefore, further research should be performed on a larger scale to better understanding the dynamics and distribution of zoonotic diseases in dogs and people with close contact with dogs (e.g., owners, animal care workers and veterinarians).



## CHAPTER X

### Conclusions and Recommendations

This thesis provides the occurrence and status of emerging and re-emerging potential zoonotic respiratory and enteric viruses in domestic dogs and humans. The genetic characteristics and diversities of potential zoonotic respiratory and enteric viruses were also elucidated by either whole genome sequencing or specific gene sequencing by using sanger and next-generation sequencing. The results of this thesis provide information for better understanding the situation, distribution and characteristics of potential zoonotic viruses at the human-dog interface. This will help the development appropriate risk communications and prevention and control of respiratory and enteric diseases in domestic dogs in Thailand. Our findings, conclusion and recommendations for each important respiratory and enteric virus in domestic dogs are provided in each chapter.

In Chapter 2, we investigated canine parainfluenza type 5 (CPIV-5) infection in dogs with respiratory symptoms from November 2015 to December 2018. This chapter supports the first and second objectives of this study which the occurrence and genetic characteristics of whole genome of CPIV-5 were obtained. This study is the first report of whole genome characterization of CPIV-5 in Thailand. Our results showed that 5.6% of nasal swab samples (32 out of 571) were positive for CPIV-5 by RT-PCR specific to the NP gene. Phylogenetic analysis showed that Thai CPIV-5 might have originated from a common ancestor with CPIV-5 from Korea and China. The result of this study is published in an international scientific journal (Tier1) in the title “Molecular detection and whole genome characterization of Canine Parainfluenza type 5 in Thailand”, 2021 Scientific Reports, 11, Article number: 3866.

In chapter 3, we investigated influenza A virus in domestic dogs from November 2015 to December 2018. This chapter also supports the first and second objectives of this study. This chapter is the first to report pandemic H1N1-2009 virus infection in domestic dogs which never been reported in Thailand. Our results showed that 1.23 % (7/571) samples were positive for influenza A virus. Our result

supported the information that pandemic H1N1 2009 infection in dogs is a reversed zoonotic transmission from human to dogs. the result of this study reported in manuscript in title Characterization of pandemic H1N1-2009 in dogs, Thailand and will be submitted to the international scientific journal.

In chapter 4, we investigated canine respiratory coronavirus (CRCoV) in dogs with respiratory signs during November 2015 to December 2018. This chapter also supports the first and second objectives of this study. The occurrence and genetic characteristics of CRCoVs were obtained by partial S, HE gene sequencing and whole genome sequencing. Our result showed that 13.13% (75/571) of nasal swabs were positive to CRCoV by using one-step RT-PCR specific to RdRp gene. Phylogenetic analysis showed that Thai CRCoVs were grouped into betacoronavirus group A with Bovine and Human coronavirus (BoV, HCoV-OC43 and HeCoV-4408) but in separated clusters from canine enteric coronavirus (CEoV) in alphacoronavirus group and canine SARS-CoV2 in betacoronavirus group B. The TMRCA analysis showed that Thai-CRCoV were estimated to separate from HCoV-OC43 and BCoV since 2004, suggesting that Thai-CRCoVs could have shared common origin with BCoV and HCoV-OC-43. It could be speculated that Thai CRCoVs potential originated from interspecies transmission between dog, human and bovine. Moreover, the nucleotide substitution rates of the Thai CRCoVs are higher than most RNA viruses. The virus with high mutation rate and can evolve and result in novel strain and subsequent cross-species transmission. The result of this study is prepared in the manuscript in title “Genetic diversity of canine respiratory coronaviruses in dogs, Thailand” and will be submitted to the international scientific journal.

In Chapter 5, we conducted a survey of canine kobuvirus (CaKoV) in Thailand during September 2016 to September 2018. CaKoVs is emerging enteric virus causing gastroenteritis and have been reported since 2000s. This chapter supports the first and second objectives of this study which the occurrence and genetic characteristics of CaKoVs were obtained by whole genome sequencing. This study was the first to report the detection and genetic characteristics of CaKoVs in domestic dogs in Thailand. In this study, we found CaKoV positivity at 17.59% (54/307). The viruses

could be detected in both healthy dogs and dogs with clinical signs. The virus is frequently detected in younger dogs. Thai CaKoVs were closely related and grouped with Chinese CaKoVs suggesting a possible origin of CaKoVs in Thailand. Our result raises a concern to vet practitioners that diarrhea in dogs due to Canine Kobuvirus infection should not be ignored. The results of this study have been published in an international scientific journal (T1) in the title “First Detection and Genetic Characterization of Canine Kobuvirus in domestic dogs in Thailand”, 2019, BMC Veterinary Research; 15, Article number: 254.

In Chapter 6, we investigated Norovirus (NoV) at the human-dog interface in a dog farm in Thailand during July-August 2018. This chapter supports all objectives (1-3) of this study. The occurrence and genetic characteristics of NoVs from dog and human samples were obtained by whole genome sequencing. Norovirus (NoV) infection is a major cause of both endemic and epidemic acute gastroenteritis in human and animals. In this study, we reported NoV infection in dogs which the virus is a human norovirus GII.4 Sydney. The NoVs infection suspected to transmit from children whom NoVs positive 2 weeks before the investigation. The disease developed in dogs and puppies after they shared the same premises and possible direct contact with the children. This observation suggested potential human-to-dog transmission of human noroviruses. Genetic and phylogenetic analyses confirmed that whole genomes of canine and human noroviruses were closely related to human norovirus GII.Pe-GII.4 Sydney. Dog owners and veterinarians should pay more attention to norovirus infection as a potential zoonotic and reverse zoonotic disease in households, animal hospitals, and shelters. The results of this study have been published in an international scientific journal (T1) in the title “Human Norovirus Infection in Dogs, Thailand” 2020, Emerging Infectious Disease;26(2):350-353.

In chapter 7, we investigated canine parvovirus type2 in domestic dogs and cats during September 2016 to April 2018. This chapter supports the first and second objectives of this study which genetic diversity and genetic characteristics of CPV-2 were obtained by specific gene and whole genome sequencing. Our results showed that the positivity of canine parvovirus (CPV) was 29.95% and that of feline parvovirus



(FPV) was 58.73% by using VP2 gene specific PCR for parvovirus. Antigenic types of CPV-2 were CPV-2c (n = 62; 46.61%), CPV-2a (n = 68; 51.13%) and CPV-2b (n = 3; 2.26%). It is noted that both CPV-2c and CPV-2a were predominant variants and CPV-2c has never been reported in Thailand. Our result showed that Thai-CPV-2c started to evolve from other Asian-CPV-2c viruses since 2004. Moreover, parvovirus (which is DNA virus) has high genomic substitution rate similar to other RNA viruses. Our findings raise a concern regarding whether currently used canine parvovirus vaccines can provide full protection against the new variant, Asian-CPV-2c. Moreover, cats can be infected with CPV-2c, dogs can also be infected with FPV. Thus, veterinary practitioners should focus more attention on both CPV and FPV infections, especially interspecies transmission. This information helps early diagnosis and the development of strategies for domestic animal vaccination. The results of this study have been published in an international scientific journal (T1) in the title “Emergence of canine parvovirus type 2c in domestic dogs and cats from Thailand” 2019, *Transboundary and Emerging Disease*; Jul;66(4):1518-1528.

In chapter 8, we investigated canine rotavirus group A (CRV) in domestic dogs in Thailand during September 2016 to January 2019. This study is the first to report on the genetics of canine rotavirus (CRV) in dogs in Thailand. This chapter supports the first and second objectives of this study which the occurrence and genetic characteristics of CRV were obtained by whole genome sequencing. Our results showed that 0.70% (5/710) were positive for CRV by using one-step RT-PCR specific to the VP6 gene. We found that the G3P[3] genotype was the predominant genotype circulating in dogs. The viruses belonged to genotype AU-1 with gene segments of both genogroup Au-1-like and Cat 97-like, which have never been reported before in any canine rotaviruses. The MCC analysis showed that Thai CRVs might originated from humans, bats and dogs through interspecies transmission and resulted in multiple reassortment of genes among humans and animals RVAs since 1965 -2002. The Thai CRVs had a higher genomic substitution rate than other dsRNA, suggesting rapid evolution of the virus. Thus, dog owners and veterinary practitioners should pay more attention to rotavirus infection as a potentially zoonotic and reverse

zoonotic viral disease. There is a need to survey CRVs on a larger scale to determine the dynamics and distribution of rotaviruses in Thailand. The results of this study have been published in an international scientific journal (T1) in the title “Molecular characterization identifies intra-host recombination and zoonotic potential of canine rotavirus among dogs from Thailand” 2020, *Transboundary and Emerging Disease*; Aug 9. doi: 10.1111/tbed.13778.

In chapter 9, we investigated potential respiratory and enteric zoonotic viruses in High-risk occupations in Bangkok, Thailand from small animal hospitals within Bangkok during June 2018 to May 2019. This chapter supports the third objectives of this study which surveillance of potential respiratory and enteric zoonotic viruses in High-risk occupations were obtained by genome sequencing and questionnaire from participants. The nasal swab samples and stool samples were collected from 100 participants for viral identification. The nasal swab samples were tested for important potential respiratory zoonosis viruses (influenza virus, parainfluenza virus and coronavirus) while stool samples were tested for important enteric zoonosis viruses (coronavirus and rotavirus). Our result of this study showed that two participants were positive for CoV, while none of the other viruses (IAV, PIV and RV) were detected. The genetic characterization of 2 positive CoV showed that the viruses were cluster with human alphacoronavirus (HCoV- 229E). Base on the questionnaire analysis showed that the most (80%) of small animal hospitals provide a safe workplace for their staff and 93% of participants use appropriate PPE when in contact with dogs. This study provided the information of potential zoonotic viruses, genetic characteristic, and gathered information on knowledge and practices that might impact zoonotic infection in dogs and humans in Bangkok. The result of this study is prepared in the manuscript in title “Surveillance of potential respiratory and enteric zoonotic viruses in High-risk occupations in Bangkok, Thailand” and will be submitted to the international scientific journal.

Our findings supported that canine respiratory and enteric viruses (CPIV-5, CIV, CRCoV, CaKoV, NoV, CPV-2 and RVA) are important pathogens causing viral diseases in domestic dogs and possibly impact on human health. There are CPIV-5, CIV

(pdmH1N1/2009), CRCoV, CaKoV, NoV (GII-P4), CPV-2 (2a, ab, ac) and RVA circulating in dog population in Thailand. Moreover, the result of this study showed that zoonotic viruses (HNoV, RVA, HCoV, CIV (pdmH1N1/2009) are circulating in dogs at human – dog interface. According to our results of this study, the recommendations for prevention and control of the viruses as following.

1. The surveillance of respiratory and enteric viruses in humans and dogs (especially high-risk occupation or pet owners) should be routinely investigated on a larger scale to determine the dynamics, distribution, and genetic characteristics of the viruses.
2. Owners/farms and pet practitioners who close contact with companion animals should be a conduct appropriate practices to reduce risk of virus transmission as following.
  - a. Good personal hygiene
  - b. Biosecurity including farm management and sanitization.
3. Herd immunity in dogs and humans
  - a. In dog, commercial vaccines (CIV, CPIV-5 and CPV-2) are available worldwide. However, the vaccination program protocol, storage, shipping and selecting appropriate type of vaccine should be concerned.
  - b. In human, our results that the evidence of reverse zoonotic (influenza virus (pdm H1N1/2009), rotavirus and norovirus) were found in dogs. The pet owners, workers, person who close contact with companion animals (veterinarians and animal care workers) should be received vaccination to reduce risk of viral transmission between humans and animals.
4. Rapid diagnosis kit could be used to routinely practice for early detection of virus disease in domestic dogs to control the outbreak of the disease.

5. The information this study is imperative to educate pet owners, practitioners who close contact with domestic dogs about zoonotic and reverse zoonotic awareness as well as the prevention and control measurements of respiratory and enteric disease in domestic dogs in Thailand.



## APPENDIX A

Molecular detection and whole genome characterization of  
Canine Parainfluenza type 5, Thailand

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## OPEN Molecular detection and whole genome characterization of Canine Parainfluenza type 5 in Thailand

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Parainfluenza virus type 5 (PIV-5) causes respiratory infection in several animal species and humans. Canine parainfluenza virus type 5 (CPIV-5) causes respiratory disease in domestic dogs worldwide. In this study, we conducted a cross-sectional survey of CPIV-5 in dogs with respiratory symptoms from small animal hospitals in Thailand from November 2015 to December 2018. Our results showed that 32 out of 571 nasal swab samples (5.6%) were positive for CPIV-5 by RT-PCR specific to the NP gene. To characterize the viruses, three representative CPIV-5 were subjected to whole genome sequencing, and an additional ten CPIV-5 were subjected to HN, F, SH and V/P gene sequencing. Pairwise sequence comparison and phylogenetic analysis showed that Thai CPIV-5 was closely related to the CPIV-5 isolated from China and Korea. In conclusion, this study constitutes a whole genome characterization of CPIV-5 from dogs in Thailand. The surveillance of CPIV-5 should be further investigated at a larger scale to determine the dynamics, distribution and potential zoonotic transmission of CPIV-5.

Parainfluenza virus (PIV) is an enveloped, nonsegmented, single-stranded RNA virus. PIV-5 belongs to the family *Paramyxoviridae*, genus *Rubulavirus*. The virus consists of seven genes encoding 8 proteins (F, HN, SH, M, NP, V, P, and L)<sup>1</sup>. PIV can be classified into 5 types, designated PIV 1–5. PIV-1 to PIV-4 can cause upper and lower respiratory tract infections in humans, especially in infants and young children<sup>2–5</sup>. PIV-5 has been reported to infect and cause respiratory disease in several host species.

PIV-5 was first isolated in 1956 from rhesus and cynomolgus monkey kidney-cells<sup>6</sup>. The virus was previously named simian virus type 5 (SV-5) according to the host of isolation. Then, SV-5 was renamed to PIV-5 and prefixed according to the isolated species<sup>7</sup>. To date, the disease caused by PIV-5 in humans are still unclear. Some studies revealed that a virus serologically related to PIV-5 was associated with multiple sclerosis (MS), sclerosing panencephalitis (SSPE), Creutzfeldt-Jakob disease (CJD), pemphigus, atherosclerosis, Paget's disease, hepatitis and common cold in humans<sup>8–10</sup>. There were in vitro studies and need to be identified as such PIV-5 was found in human respiratory cells and might impact human respiratory diseases<sup>11,12</sup>.

PIV-5 has been reported in several host species including pigs, cattle, dogs, hamsters, ferrets, monkeys, calves, lesser pandas and guinea pigs<sup>10,13,14</sup>. In pigs, PIV-5 co-infects with porcine reproductive and respiratory syndrome (PRRSV) and causes respiratory symptoms. In cattle, PIV-5 possibly causes severe respiratory illness and leads to a high morbidity rate in calves<sup>15</sup>. In dogs, canine parainfluenza virus type 5 (CPIV-5) was first isolated from dogs with respiratory signs in 1967 and was first named canine parainfluenza virus type 2 (CPIV-2) due to it causing a respiratory disease similar to that of human parainfluenza type 2 (HPIV-2)<sup>16</sup>. A subsequent study based on antigenic and sequence analyses revealed that CPIV-5 and HPIV-2 are different<sup>17</sup>. It has been reported that CPIV-5 is one of the common pathogens of canine infectious respiratory disease (CIRD). CPIV-5 causes mild to moderate respiratory illness in dogs. Dogs can develop severe clinical signs if co-infected with other respiratory viruses or bacteria<sup>18–20</sup>. In some cases, CPIV-5 can cause neurological disorders especially in puppies including encephalitis, seizures, myoclonus and posterior paresis<sup>21,22</sup>. The cross-species transmission of CPIV-5 has been reported in coyotes, ferrets and rodents<sup>23,24</sup>.

Interspecies transmission of PIV-5 between canines and humans has not been reported. However, a study suggested that PIV-5 might be a potential zoonotic pathogen<sup>25</sup>. Some studies have supported the hypothesis that genetic characteristics between PIV-5 isolated from canines and humans are highly similar with fewer nucleotide

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Virus	Collection date	Age	Breed	Vaccination history	CPIV-5 detection	Sequencing	# GenBank
CU-D58	Jan 16	3 mts	Siberian Husky	I	+	F, HN, SH, V/P <sup>a</sup>	MT604002-05
CU-D103	Feb 16	2 mts	Bully	I	+	F, HN, SH, V/P	MT604006-09
CU-D133	Apr 16	>7 years	Golden retriever	C	+	WGS <sup>b</sup>	MT603999
CU-D151	May 16	3 mts	Pomeranian	I	+	WGS	MT604000
CU-D373	Nov 16	3 mts	Pomeranian	I	+	F, HN, SH, V/P	MT604011-13
CU-D376	Dec 16	>1 year	Mixed	I	+	F, HN, SH, V/P	MT604014-17
CU-D381	Dec 16	3 mts	Pekingese	I	+	F, HN, SH, V/P	MT604018-21
CU-D399	Jan 17	4 mts	Pomeranian	I	+	F, HN, SH, V/P	MT604022-25
CU-D400	Jan 17	7 mts	Pomeranian	I	+	F, HN, SH, V/P	MT604026-29
CU-D406	Jan 17	3 mts	Pomeranian	I	+	F, HN, SH, V/P	MT604030-33
CU-D466	Mar 17	2 mts	Mixed	I	+	F, HN, SH, V/P	MT604034-37
CU-D585	Sep 17	3 mts	Mixed	I	+	F, HN, SH, V/P	MT604038-41
CU-D20804	Feb 18	4 mts	Mixed	I	+	WGS	MT604001
CU-D361	Oct 16	2 mts	Pomeranian	I	+	–	–
CU-D369	Nov 16	6 mts	Pomeranian	I	+	–	–
CU-D370	Nov 16	10 years	Shih-Tzu	C	+	–	–
CU-D371	Nov 16	12 years	Poodle	C	+	–	–
CU-D372	Nov 16	4 mts	Pomeranian	I	+	–	–
CU-D377	Dec 16	>1 year	Mixed	I	+	–	–
CU-D380	Dec 16	10 mts	Mixed	I	+	–	–
CU-D390	Dec 16	>5 years	Mixed	C	+	–	–
CU-D483	Mar 17	>1 year	Mixed	I	+	–	–
CU-D489	Mar 17	>1 year	Mixed	I	+	–	–
CU-D493	Mar 17	6 mts	Mixed	I	+	–	–
CU-D497	Mar 17	6 mts	Mixed	I	+	–	–
CU-D20273	Dec 17	2 mts	Pomeranian	I	+	–	–
CU-D20277	Dec 17	2 mts	Bully	I	+	–	–
CU-D20364	Dec 17	3 mts	Pomeranian	I	+	–	–
CU-D20384	Jan 18	3 mts	Mixed	I	+	–	–
CU-D20803	Feb 18	3 mts	Mixed	I	+	–	–
CU-D21496	May 18	2 mts	Mixed	I	+	–	–
CU-D22309	Sep 18	3 mts	Samoyed	I	+	–	–

**Table 1.** Description of canine parainfluenza type 5 (CPIV-5) characterized in this study. C complete vaccination, I incomplete vaccination. <sup>a</sup>F, HN, SH, V/P; F, HN, SH, V/P gene sequencing. <sup>b</sup>WGS; whole genome sequencing.

sequence variations (only 0.1% to 3% nucleotide difference)<sup>7,26,27</sup>. In addition, CPIV-5 can be grown in various cell lines especially human cell lines (2FTGH and HEp2) which might correlate with the host preference of the virus<sup>28</sup>. Since epidemiological and whole genome sequence information on CPIV-5 is still limited, in this study, we conducted a cross-sectional survey of CPIV-5 in dogs and characterized the whole genome of Thai CPIV-5.

## Results

In this study, we investigated canine parainfluenza type 5 (CPIV-5) infection in dogs with respiratory symptoms from November 2015 to December 2018. Our results showed that 5.6% (32/571) of nasal swab samples were positive for CPIV-5. From 3 years of surveillance, the highest occurrence of CPIV-5 was observed in November 2016 (41.7%), followed by December 2016 (33.3%) with statistical significance  $p < 0.05$  when compared to other years (Supplement Table S1). Regarding the relationship between CPIV-5 infection and age group, the occurrences of CPIV-5 was statistically more frequent in dogs < 1 year (10.0%; 24/240) than in dogs older than 5 years (3.3%; 4/120) and dogs 1–4 years (1.9%; 4/211) ( $p = 0.0349$  and  $0.0003$ ,  $p < 0.05$ ), respectively. Regarding the relationship between CPIV-5 infection and vaccination history, the occurrence of CPIV-5 infection in dogs with incomplete CPIV-5 vaccination (10.4%; 28/269) was higher than in dogs fully vaccinated (1.3%; 4/302), with statistical significance ( $p < 0.05$ ).

**Genetic characteristics of Thai canine parainfluenza type 5.** In this study, we selected and characterized representatives of Thai CPIV-5 for whole genome sequencing ( $n = 3$ ; CU-D133, CU-D151 and CU-D20804) and F, HN, V/P and SH gene sequencing ( $n = 10$ ) (Table 1). Our results showed that the genome size of Thai CPIV-5 is 15,207 bp, containing seven genes as 3'-N-V/P-M-F-SH-HN-L-5'. Whole genome sequence analysis showed that Thai CPIV-5 possessed high nucleotide identity to the reference PIV5 with 96.1–99.4% nucleotide

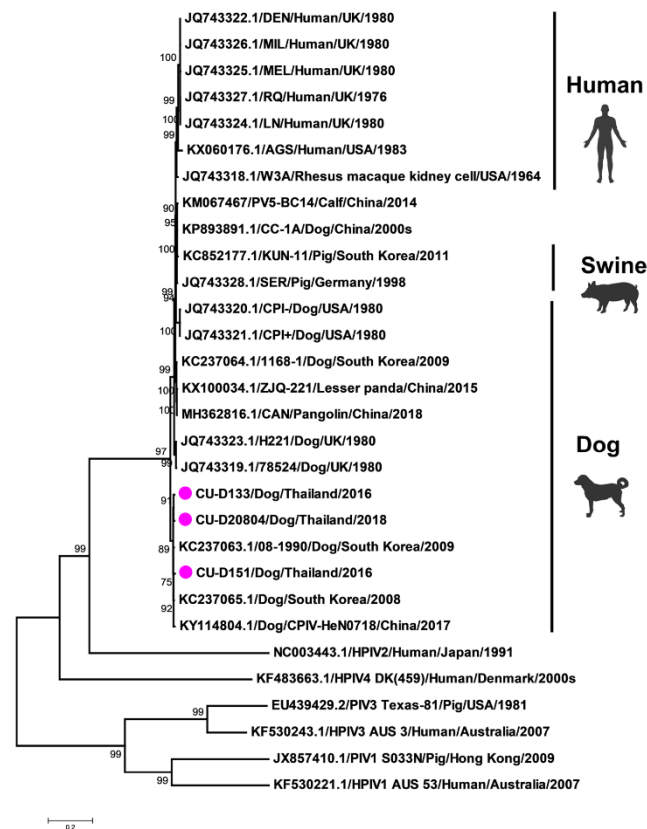
Virus	Accession no.	Host	Location	(% Nucleotide identity)								
				WGS	N (1530 nt)	F (1590–1656 nt)	HN (1698 nt)	SH (135 nt)	V (669 nt)	P (1177 nt)	M (1134 nt)	L (6768 nt)
CU-D151	This study	Canine	Thailand	100.0	100.0	100.0	100.0	(–)	100.0	100.0	100.0	100.0
CU-D133	This study	Canine	Thailand	99.1	99.3	99.0	99.5	(–)	99.0	98.9	99.4	99.3
CU-D20804	This study	Canine	Thailand	99.2	99.5	99.4	98.8	(–)	99.3	99.2	99.1	99.2
CU-D58	This study	Canine	Thailand	(–)	99.5	99.5	99.2	(–)	99.3	99.2	(–)	(–)
CU-D103	This study	Canine	Thailand	(–)	99.3	99.0	99.6	(–)	98.8	98.8	(–)	(–)
CU-D373	This study	Canine	Thailand	(–)	(–)	99.2	97.2	(–)	99.0	99.1	(–)	(–)
CU-D376	This study	Canine	Thailand	(–)	(–)	97.3	99.9	(–)	99.3	99.2	(–)	(–)
CU-D381	This study	Canine	Thailand	(–)	(–)	99.5	99.8	(–)	99.3	99.2	(–)	(–)
CU-D399	This study	Canine	Thailand	(–)	(–)	96.8	98.8	(–)	97.2	97.0	(–)	(–)
CU-D400	This study	Canine	Thailand	(–)	(–)	99.1	99.2	(–)	99.0	99.1	(–)	(–)
CU-D406	This study	Canine	Thailand	(–)	(–)	99.5	99.4	(–)	99.3	99.2	(–)	(–)
CU-D466	This study	Canine	Thailand	(–)	(–)	99.3	99.5	(–)	99.1	99.2	(–)	(–)
CU-D585	This study	Canine	Thailand	(–)	(–)	99.3	100.0	(–)	99.1	99.2	(–)	(–)
Reference PIV-5												
AGS	KX060176	AGS cell	USA	96.1	96.2	95.0	95.8	(–)	95.7	95.9	95.6	96.7
DEN	JQ743322	Human	UK	96.6	96.6	95.6	96.4	(–)	96.1	96.1	96.0	97.0
MIL	JQ743326	Human	UK	96.5	96.5	95.6	96.3	(–)	96.0	96.0	96.0	97.0
MEL	JQ743325	Human	UK	96.5	96.3	92.5	96.4	(–)	96.1	96.1	95.9	97.0
RQ	JQ743327	Human	UK	96.5	96.5	95.5	96.3	(–)	96.0	96.0	95.9	97.0
LN	JQ743324	Human	UK	96.5	97.2	95.5	96.3	(–)	96.0	96.0	95.9	97.0
W3A	JQ743318	Macaque cell	USA	97.0	97.3	92.0	96.9	(–)	96.9	96.7	95.9	97.6
HeN0718	KY114804	Canine	China	99.2	99.5	99.3	96.9	(–)	98.8	99.1	99.0	99.3
CC-14	KP893891	Canine	China	97.2	97.5	96.4	97.6	(–)	96.7	96.9	96.3	97.8
H221	JQ743323	Canine	UK	97.5	97.5	96.7	97.9	(–)	97.6	97.2	96.9	98.1
78524	JQ743319	Canine	UK	97.5	97.4	96.7	97.9	(–)	97.3	97.1	96.9	97.9
CPI+	JQ743321	Canine	USA	96.7	96.4	95.6	96.9	(–)	96.3	96.3	95.9	97.3
CPI-	JQ743320	Canine	USA	96.7	96.4	95.5	96.9	(–)	96.0	96.2	95.9	97.3
08-1990	KC237063	Canine	Korea	99.2	99.5	99.5	99.5	(–)	99.4	99.3	99.5	99.4
D277	KC237065	Canine	Korea	99.4	99.9	99.5	99.6	(–)	99.6	99.6	99.6	99.6
1168-1	KC237064	Canine	Korea	97.4	97.1	96.6	97.9	(–)	97.0	96.9	96.9	98.0
SER	JQ743328	Swine	Germany	97.2	97.3	96.4	97.6	(–)	96.7	96.9	96.4	97.7
KNU-11	KC852177	Swine	Korea	97.0	96.9	96.3	97.3	(–)	96.1	96.3	96.0	97.6
PV5-BC14	KM067467	Calve	China	97.2	97.1	96.4	97.5	(–)	96.6	96.7	96.4	97.7
ZJQ-221	KX100034	Lesser panda	China	97.3	96.9	96.5	97.6	(–)	97.0	96.9	96.7	97.9
Other reference PIV 1 to IV												
HPIV-1	KF530221	Human	Australia	45.9	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
S033N	JX857410	Swine	Hong Kong	44.5	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
HPIV-2	NC003443	Human	Japan	63.1	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
HPIV-3	NC001796	Human	Australia	46.8	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
Texas-81	EU439429	Swine	USA	45.8	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
HPIV-4	KF483663	Human	Denmark	52.1	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)

**Table 2.** Pairwise comparison of whole genome nucleotide sequences of Thai CPIV-5 (CU-D151) with reference parainfluenza viruses.

identities but low percentages of nucleotide identities with PIV-1 to PIV-4 (44.5–63.1% nucleotide identities). Comparing PIV-5, the whole genome of Thai CPIV-5 was closely related to Chinese CPIV-5 (HeN0718, 99.2% nucleotide identities) and Korean CPIV-5 (D277 and 08-1990, 99.4% and 99.2% nucleotide identities) (Table 2). For phylogenetic analysis, Thai CPIV-5 (n = 3) was grouped with PIV-5 from humans, pigs, dogs, lesser panda, and pangolins but separated from clusters of PIV-1 to PIV-4. The phylogenetic tree of the whole genome of PIV-5 could be divided into subgroups, e.g., human and simian subgroup, cattle and swine subgroup and canine subgroup. Thai CPIV-5 was grouped in the canine subgroup with CPIV-5 from China (HeN0718) and Korea (D277 and 08-1990) (Fig. 1).

Pairwise comparison of nucleotide sequences showed that the HN, F, V/P and SH genes of Thai CPIV-5 possessed high nucleotide identities to Chinese CPIV-5 (HeN0718; 96.9–99.5%) and Korean CPIV-5 (D277 and 08-1990; 99.3–99.9%), which were similar to the whole genome sequences (Table 2). The phylogenetic analysis

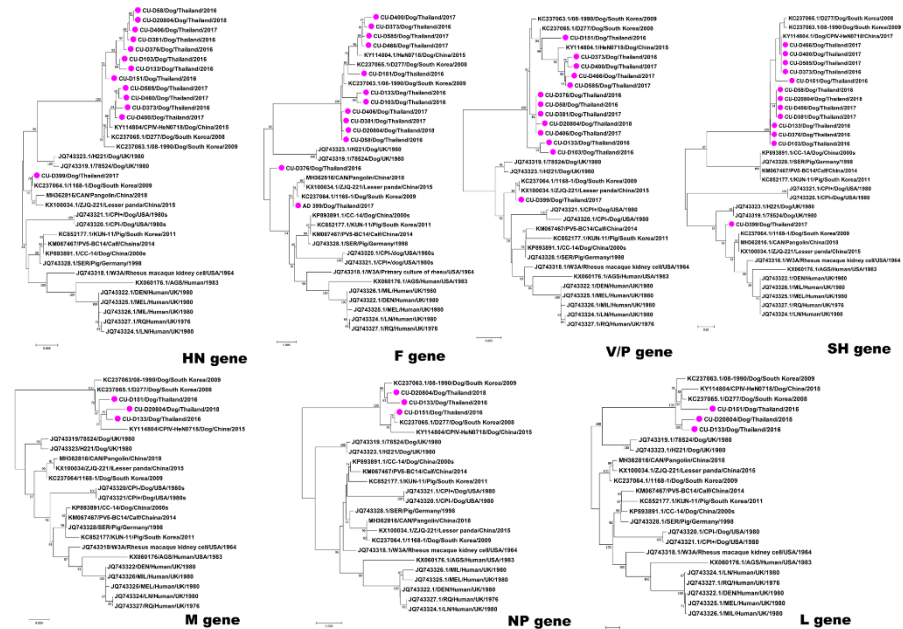




**Figure 1.** Phylogenetic tree of the whole genome of Thai CPIV-5 and reference PIV-5. Pink circles indicate Thai CPIV-5 in this study. The phylogenetic tree was constructed by using MEGA v.7.0 (Tempe, AZ, USA) with the neighbor-joining method with the Kimura 2-parameter with 1000 bootstrap replication<sup>52</sup>. The drawing was created by professional science figure service (BioRender.com).

of the E, HN, and V/P genes showed that Thai CPIV-5 was grouped with Chinese CPIV-5 (HeN0718) and Korea CPIV-5 (D277 and 08-1990) (Fig. 2). Moreover, the M, NP and L genes of Thai CPIV-5 (CU-D131, CU-D151 and CU-D20804) had the highest nucleotide identities to Korean CPIV-5 (D277; 99.6–99.9%). The phylogenetic analysis results showed that the M, NP and L genes were also closely related to CPIV-5 from Chinese and Korean strains (Fig. 2).

Genetic analysis of the HN gene (1698 nucleotides, 565 amino acids) of Thai CPIV-5 showed that amino acid residues at the receptor binding site (positions 186–190) and cleavage site (positions 390 and 523) of the HN protein contained QDHVS (186–190), E390 and Y523. Amino acid residues at the stalk regions contained S60, Y77, I90, E91 and Q102 identical to the reference PIV-5. Amino acid residues at positions 37, 342, 437, and 457, which correlated with neutralizing antibodies, contained E37, K342, T437, and F457. It is noted that Q342K was only observed in all Thai CPIV-5, which was identical to CPIV-5 from China (HeN0718) and Korea (D277 and 08-1990) but not in other CPIV-5 and human PIV-5 (Table 3). Amino acid residues related to host preference (human specific) at I22L, A49S, R57G, T254A, N318S, K460T and M536T were analyzed. Thai CPIV-5 contained I22, A49, R57, T254, N318, K460 and M536, which are not human specific amino acids. Unique amino acids for Thai, Chinese and Korean CPIV-5 were also observed at T19I, K43E, T62I, T141A, F252L, F353L and G446R suggesting unique subclustered characteristics (Table 4).



**Figure 2.** Phylogenetic trees of the HN, F, M, V/P, NP, and L genes of Thai CPIV-5 and reference PIV-1-5. Pink circles indicate Thai CPIV-5 in this study. The phylogenetic tree was constructed by using MEGA v.7.0 (Tempe, AZ, USA) with the neighbor-joining method with the Kimura 2-parameter with 1000 bootstrap replication<sup>32</sup>.

Genetic analysis of the F gene showed a low level of genetic variation. Amino acid residues related to host preference (human specific) were observed at T31, S19G, I301M, T438S, I498R, S530Q and R536Q. One Thai CPIV-5 (CU-D151) contained R536Q similar to some human PIV-5 (DEN, MIL, RQ, and LN). Moreover, Thai CPIV-5 contained 22P and 443P, which were similar to PIV-5 from humans and pigs suggesting potential human preference characteristics<sup>29–31</sup> (Supplement Table S2). Genetic analysis of the V/P gene showed that amino acids related to viral RNA synthesis contained S157, T286 and K254 similar to most CPIV-5 (Supplement Table S3).

Genetic analysis of the SH gene showed that Thai CPIV-5 (CU-D58, CU-D103, CU-D133, CU-D151, CU-D376, CU-D381, CU-D406, and CU-D20804) contained a non-synonymous substitution at the start codon (MIT). Distinct nucleotide substitutions at T133C were observed and resulted in the extension of four amino acids at the stop codon, similar to those of CPIV-5 from China and Korea. Thus, the SH protein of Thai, Chinese, and Korean CPIV-5 is four amino acids longer than that of the reference PIV-5 (Supplement Table S3 and Fig. 3).

## Discussion

Parainfluenza virus type 5 (PIV-5) can infect and cause respiratory diseases in various mammals. Canine parainfluenza virus type 5 (CPIV-5) is highly contagious and causes mild to moderate respiratory diseases in dogs worldwide. Coinfection with CPIV-5 and other viruses or bacteria can cause more virulent clinical signs. This study revealed the occurrence of CPIV-5, which was relatively high during the winter season in Thailand (November to January). A similar finding of high occurrence detected in the cold season has also been reported<sup>32</sup>. CPIV-5 could be detected in younger dogs (< 1 year) more than in older dogs. Dogs of all ages could be infected with CPIV-5, but younger dogs (< 1 year) are more susceptible. This observation is in agreement with a previous report that CPIV-5 could be observed more in younger dogs than in dogs in other age groups<sup>33–35</sup>. Regarding vaccination history, CPIV-5 infection was higher in dogs with incomplete vaccination (10.4%) than in dogs with complete vaccination (1.32%). The CPIV-5 vaccine used in Thailand was modified live CPIV-5 combined with other pathogens (e.g., canine distemper virus, canine parvovirus and canine coronavirus). Some studies have suggested that vaccinated dogs can show mild clinical signs and shed the virus after infection<sup>36</sup>. It is noted that, the CPIV-5 characterized in this study was obtained from nasal swabs of dogs with and without vaccination. A previous study revealed that whole genome sequences of CPIV-5 vaccine was identical with PIV-5 strain W3A, which different from Thai-CPIV-5<sup>37</sup>. Moreover, all three Thai-CPIV-5 contained unique amino acids of Asian

Virus	Host	HN gene											
		HN gene				Receptor binding site 186-190	Cleavage site		HN stalk				
		37	342	437	457		390	523	60	77	90	91	102
<b>Reference PIV-5</b>													
AGS	AGS cell	E	K	T	A	QDHVS	E	Y	S	Y	L	E	H
W3A	Macaque cell	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
DEN	Human	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
MIL	Human	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
MEL	Human	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
RQ	Human	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
IN	Human	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
SER	Swine	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
KNU-11	Swine	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
PV5-BC14	Calve	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
ZJQ-221	Lesser panda	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
CAN	Pangolin	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
H221	Canine	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
78524	Canine	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
CPI+	Canine	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
CPI-	Canine	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
08-1990	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
D277	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
1168-1	Canine	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
CC-14	Canine	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
HeN0718	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
<b>This study</b>													
CU-D58	Canine	E	K	I	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D103	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D133	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D151	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D373	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D376	Canine	E	K	I	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D381	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D399	Canine	E	Q	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D400	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D406	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D466	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D585	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q
CU-D20804	Canine	E	K	T	F	QDHVS	E	Y	S	Y	L	E	Q

**Table 3.** Genetic analysis of the HN gene of Thai CPIV-5 and reference PIV-5 at the receptor binding, cleavage site and stalk region.

CPIV-5 subcluster and distinguished from W3A and CPIV-5 from the US and UK. Thus, it more likely that the CPIV-5 in this study were isolated from naturally infected dogs in Thailand.

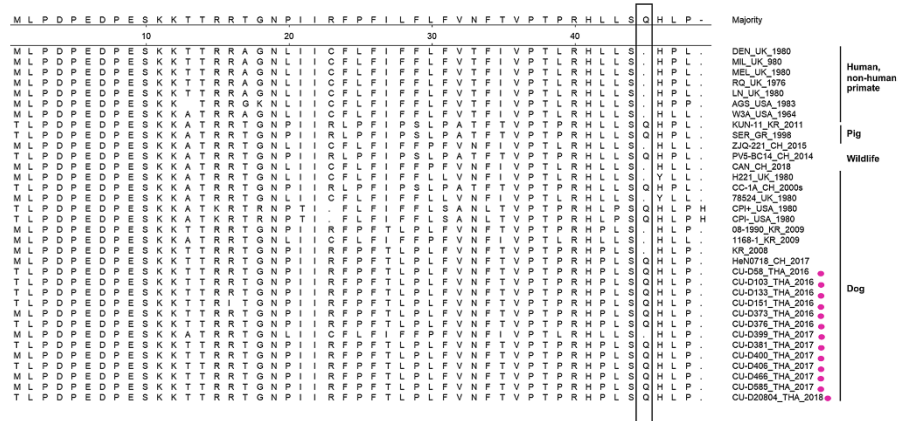
To date, only nine whole genome sequences of CPIV-5 are available in the GenBank database. This study provided additional information on the whole genome sequences of CPIV-5 from Thailand ( $n = 3$ ). Based on phylogenetic analysis of the whole genome, Thai CPIV-5 belongs to parainfluenza type 5 and subcluster CPIV-5 (canine sublineage) and is separated from swine and human sublineage. Within the canine sublineage, Thai CPIV-5 was closely related to CPIV-5 from South Korea (08-1990 and D227) and China (CPIV-HeN0718). Thai CPIV-5 had the highest nucleotide identities (99.4%) to CPIV-5 from Korea. Phylogenetic analyses of the HN, F, V/P, M, NP and L genes showed similar results, in which Thai CPIV-5 was grouped together with CPIV-5 from Korea (08-1990 and D227) and China (CPIV-HeN0718). One Thai CPIV-5 (CU-D399) was closely related to PIV-5 from the pangolin (CAN) and lesser panda (ZJQ-221), which was similar to CPIV-5 (1168-1 from Korea). Our results suggested that Thai CPIV-5 potentially originated from the same ancestor as CPIV-5 from China and South Korea. Similarly, a unique cluster of CPIV-5 from dog in China (CC-1A, 2000s), PIV-5 from calf in China (PV5-BC14, 2014) and PIV-5 from pig in Germany (SER, 1998) and South Korea (KUN-11, 2011) was observed suggesting potential common ancestor of these viruses and required further investigations.

Virus	Host	Location	Primate specific amino acid						Lineage specific amino acid <sup>a</sup>							
			22	49	57	254	318	460	536	19	43	62	141	252	353	446
<b>Reference PIV-5</b>																
AGS	AGS Cell		L	S	G	A	S	T	T	T	K	T	T	F	F	G
DEN	Human	UK	L	S	G	A	S	T	T	T	K	T	T	F	F	G
MIL	Human	UK	L	S	G	A	S	T	T	T	K	T	T	F	F	G
MEL	Human	UK	L	S	G	A	S	T	T	T	K	T	T	F	F	G
RQ	Human	UK	L	S	G	A	S	T	T	T	K	T	T	F	F	G
LN	Human	UK	L	S	G	A	S	T	T	T	K	T	T	F	F	G
W3A	Macaque cell		I	A	R	A	N	T	M	T	K	T	T	F	F	G
SER	Swine	Germany	I	A	R	T	N	K	M	T	K	T	T	F	F	G
KNU-11	Swine	South Korea	I	A	R	T	N	K	M	T	K	T	T	F	F	G
PV5-BC14	Calve	China	I	A	R	T	N	K	M	T	K	T	T	F	F	G
ZJQ-221	Lesser panda	China	I	A	R	T	N	K	M	T	K	T	T	F	F	G
CAN	Pangolin	China	I	A	R	T	N	K	M	T	K	T	T	F	F	G
H221	Canine	UK	I	A	R	T	N	K	I	T	K	T	T	F	F	G
78524	Canine	UK	I	A	R	T	N	K	M	T	K	T	T	F	F	G
CPI+	Canine	USA	I	A	R	T	N	K	M	T	K	T	T	L	F	G
CPI-	Canine	USA	I	A	R	T	N	K	M	T	K	T	T	L	F	G
08-1990	Canine	South Korea	I	A	R	T	N	K	M	I	E	I	A	L	L	R
D277	Canine	South Korea	I	A	R	T	N	K	M	I	E	I	A	L	L	R
1168-1	Canine	South Korea	I	A	R	T	N	K	M	T	K	T	T	F	F	G
CC-14	Canine	China	I	A	R	T	N	K	I	T	K	T	T	F	F	G
HeN0718	Canine	China	I	A	R	T	N	K	M	I	E	I	A	L	L	R
<b>This study</b>																
CU-D58	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D103	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D133	Canine	Thailand	I	A	R	T	N	K	I	I	E	I	A	L	L	R
CU-D151	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D373	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D376	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D381	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D399	Canine	Thailand	I	A	R	T	N	K	M	T	K	T	T	F	F	G
CU-D400	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D406	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D466	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D585	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R
CU-D20804	Canine	Thailand	I	A	R	T	N	K	M	I	E	I	A	L	L	R

**Table 4.** Genetic analysis of the HN gene of Thai CPIV-5 and reference PIV-5 at the human-specific residues. <sup>a</sup>Lineage: CPIV-5 sublineage; Thai, Chinese, and Korean sublineages.

Thai CPIV-5 contained no amino acid mutations in the HN protein at the receptor binding site, cleavage site or HN stalk region. It has been reported that the amino acid residue at E37 is associated with virus entry into host cells by clathrin-coated pits and the endocytic pathway<sup>38,39</sup>. Amino acid residues at L90, E91, Q102, QDHVS (186-190), E390 and Y523 are associated with viral receptor binding of the viruses<sup>40,41</sup>. Amino acid residues at K342, T437, and F457 are associated with neutralizing antibodies<sup>42</sup>. In this study, some Thai CPIV-5 contained T437I (CU-D58 and CU-D376) and K342Q (CU-D399) which is similar to PIV-5 from dogs and humans. However, the importance of these mutations (T437I and K342Q) in neutralizing antibodies requires further investigation. A previous study reported that mutations in HN stalk regions might affect viral fusion to host cells<sup>41,43</sup>.

Thai CPIV-5 contained some host preference amino acid residues (human specific residues) in the F gene. For example, the amino acids at 22P and 443P in the F gene were observed in both Thai CPIV-5 and PIV-5 from humans and pigs<sup>29-31</sup>. One Thai CPIV-5 (CU-D151) also contained R536Q, similar to human PIV-5. For the V and P proteins, there was no amino acid mutation in Thai CPIV-5. It has been reported that amino acid mutations of S157F, K254 R and T286A of V and P proteins can result in high progeny virus production and the apoptosis of infected cells<sup>44-46</sup>. For the SH protein, Thai CPIV-5 contained an amino acid substitution at the start codon, which can also be observed in swine PIV-5, cattle PIV-5 and canine PIV-5. Mutation of the start codon can result in no expression of the SH protein<sup>7,31</sup>. The function of the SH protein is unclear, but some studies have reported an association with virus survival in host cells and control of host cell apoptosis<sup>31,47,48</sup>. It should be noted that



**Figure 3.** Alignment of deduced amino acids of the SH gene of Thai CPiV-5 and reference PIV-5 viruses. The box indicates amino acid substitution at the stop codon (Q). Pink circles indicate Thai CPiV-5 in this study.

Thai, Chinese and Korean CPiV-5 contained four amino acids longer than the reference PIV-5. Thus, the SH gene can be used as a genetic marker for the differentiation of Asian CPiV-5 from other CPiV-5.

In summary, this study is the first report of whole genome characterization of CPiV-5 in Thailand. Phylogenetic analyses showed that Thai CPiV-5 might have originated from a common ancestor with CPiV-5 from Korea and China. To date, there is no evidence of PIV-5 cross-species transmission between dogs and humans. However, it is imperative to educate pet owners, veterinarians and others who come into close contact with domestic dogs about zoonotic awareness. In Thailand, the surveillance of CPiV-5 should be further investigated on a larger scale to determine the dynamics, distribution and genetic characteristics of CPiV-5.

**Materials and methods**

**Canine samples.** From November 2015 to December 2018, a total of 571 nasal swab samples were collected from dogs with respiratory symptoms, including sneezing, nasal discharge, cough, and dyspnea. Sample collection was conducted at Chulalongkorn University’s Veterinary Teaching Hospital and private small animal hospitals in Bangkok, Thailand. The animal demographic data, including age, sex, breed, contact history, and vaccination history, were recorded. This study was conducted under approval from the Institute of Animal Use and Care Committee (IACUC# 1731074), and all procedures were completed in accordance with the relevant guidelines and regulations.

**Canine parainfluenza virus identification.** RNA extraction from nasal swab samples was conducted by using the QIAmp viral RNA mini kit (Qiagen, Hilden, Germany) following the manufacturer’s recommendations. Briefly, 140 µl of nasal swab sample was lysed by Buffer AVL-carrier RNA and 560 µl of ethanol. The mixture was centrifuged and transferred into a column, and then 500 µl each of buffers AW1 and AW2 were added. Finally, the RNA was eluted by 50 µl of buffer AVE. RNA was stored at -20 °C until use. CPiV-5 detection was performed by using a nested RT-PCR assay specific to the NP gene of PIV-5 (Supplement Table S4)<sup>49</sup>. Briefly, one-step nested RT-PCR was conducted in a total final volume of 25 µl comprised of 3 µl of template RNA, 12.5 µl of 2× reaction mix, 0.6 µl of 10 µM forward (CPiV-F363) and reverse primer (CPiV-R538), 1.2 µl of SuperScript III RT (Invitrogen, USA) and distilled water to a final volume of 25 µl. The first round of PCR product was diluted 1:5 with distilled water and subjected to a second round by using the TopTaq Master Mix Kit (Qiagen, Germany). The final volume was 20 µl, including 10 µl of 2× TopTaq Master Mix, 1 µl of 10 µM forward (CPiV-F428) and reverse primer (CPiV-R538), 2 µl of 10× coral load, and 1 µl of DNA. For the first round of nested RT-PCR conditions, the reaction contained a cDNA synthesis step at 55 °C for 30 min, an initial denaturation step at 94 °C for 2 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 68 °C for 30 s, and a final extension step at 68 °C for 6 min. For the second round of nested PCR conditions, the reaction comprised an initial denaturation step at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s and a final extension step at 72 °C for 7 min. To confirm CPiV5, 4 µl of PCR product was run on a 1.5% agarose gel with red safe. The expected size of the positive CPiV-5 product was 188 bp. Statistical analysis by the Fisher’s exact test was used to compare the proportion of CPiV-5 positivity among dogs categorized by the time of sample collection, age of dogs, and vaccination history.



**Canine parainfluenza virus isolation.** To isolate CPIV-5, RT-PCR-positive nasal swabs were subjected to virus isolation by using a Vero cell monolayer (ATCC, USA) at the Faculty of Veterinary Science, Chulalongkorn University. In brief, nasal swab sample were filtered with a 0.22  $\mu\text{m}$  filter and inoculated onto a Vero cell monolayer containing Dulbecco's minimal essential medium (DMEM, Gibco), 2% fetal bovine serum (FBS, Gibco), and gentamycin sulfate (50  $\mu\text{g}/\text{ml}$ ) at 37  $^{\circ}\text{C}$  in 5%  $\text{CO}_2$ . If a cytopathic effect (CPE) was observed, the virus was harvested by centrifugation at 1000 rpm for 10 min. The cell suspension was then screened for CPIV-5 by using nested RT-PCR as previously described<sup>49,50</sup>. The isolated viruses were kept at  $-80^{\circ}\text{C}$  for the pathogenesis studies in the future.

**Canine parainfluenza virus characterization.** In this study, Thai-CPIV-5 was selected for either whole genome sequencing (n=3) or F, HN, V/P, and SH gene sequencing (n=10). The representative CPIV-5 was selected based on epidemiological and demographic data such as the age of the dog, date of isolation, breed, and vaccination history. For whole genome sequencing, nucleotide sequences of each virus gene were amplified by PCR using oligonucleotide primers specific to each gene. The primers were synthesized per previous report and newly designed by using Primer 3 plus (Supplement Table S4)<sup>50,51</sup>. Nucleotide sequencing was conducted at the 1<sup>st</sup> Base Laboratories Sdn Bhd, Malaysia. The nucleotide sequences were validated and assembled by SeqMan software v.5 v.5.03 (DNASTAR Inc., Wisconsin, USA). In this study, nucleotide sequences of Thai CPIV-5 were submitted to the GenBank database under the accession numbers MT603999-MT604041 (Table 1).

Phylogenetic and genetic analyses were carried out by comparing nucleotide sequences of Thai CPIV-5 with those of PIV-5 available from the GenBank database. The reference nucleotide sequences of PIV-5 were retrieved based on geographic location, and host species including human PIV-1 (KF530221), swine PIV-1 (S033N; JX857410), human PIV-2 (NC003443), human PIV-3 (NC001796), swine PIV-3 (Texas-81; EU439429), and human PIV-4 (KF483663). Reference PIV-5 includes human strains (AGS; KX060176, DEN; JQ743322, MIL; JQ743326, MEL; JQ743325, RQ; JQ743327, LN; JQ743324), a rhesus macaque kidney cell strain (W3A; JQ743318.1), canine strains (HeN0718; KY114804, CC-14; KP893891, H221; JQ743323, 78524; JQ743319, CPI+; JQ743321, CPI-; JQ743320, 08-1990; KC237063, D277; KC237065, 1168-1; KC237064), swine strains (SER; JQ743328, KNU-11; KC852177), a cattle strain (PVS-BC14; KM067467), a lesser panda strain (ZJQ-221; KX100034) and a pangolin strain (CAN; MH362816). Phylogenetic analysis of CPIV-5 was performed by using MEGA v.7.0 (Tempe, AZ, USA) with the neighbor-joining method with the Kimura 2-parameter with 1,000 bootstrap replicates<sup>52</sup>. For genetic analysis, the nucleotide sequences and deduced amino acids of CPIV-5 were aligned and compared using MegAlign software v.5.03 (DNASTAR Inc., Wisconsin, USA).

**Statistical analysis.** Categorical data corresponding to the time of sample collection, age of dogs, and vaccination history were analyzed using the Fisher's exact test (<https://www.socscistatistics.com/tests/fisher/>). A p-value of <0.05 was considered as statistically significant.

**Ethics statement.** This study was conducted under the approval of the Institute for Animal Care and Use Protocol of the CU-VET, Chulalongkorn University (IACUC # 1731074).

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### Author contributions

K.C., C.N., R.T., N.B. and S.B. performed sample collection, molecular detection, whole genome characterization and analysis. C.N., S.C. and T.J. participated in whole genome sequencing and phylogenetic analysis. K.C. drafted the manuscript. A.A. (PI) designed the study, performed data analysis, drafted, revised and approved the manuscript. All authors reviewed the manuscript.

### Completing interests

The authors declare no competing interests.


### Additional information

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## APPENDIX B

**First detection and genetic characterization of canine Kobuvirus in domestic dogs in Thailand**

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**Keywords:** Canine, Characterization, Detection, Kobuvirus, Thailand

## RESEARCH ARTICLE

## Open Access

# First detection and genetic characterization of canine Kobuvirus in domestic dogs in Thailand



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## Abstract

**Background:** Canine Kobuvirus (CaKoV) has been detected both in healthy and diarrheic dogs and in asymptomatic wild carnivores. In this study, we conducted a survey of CaKoV at small animal hospitals in Bangkok and vicinity of Thailand during September 2016 to September 2018.

**Results:** Three hundred and seven rectal swab samples were collected from healthy dogs ( $n = 55$ ) and dogs with gastroenteritis symptoms ( $n = 252$ ). Of 307 swab samples tested by using one-step RT-PCR specific to 3D gene, we found CaKoV positivity at 17.59% (54/307). CaKoVs could be detected in both sick (19.44%) and healthy (9.09%) animals. In relation to age group, CaKoV could be frequently detected in younger dogs (25.45%). Our result showed no seasonal pattern of CaKoV infection in domestic dogs. In this study, we characterized CaKoVs by whole genome sequencing ( $n = 4$ ) or 3D and VP1 gene sequencing ( $n = 8$ ). Genetic and phylogenetic analyses showed that whole genomes of Thai CaKoVs were closely related to Chinese CaKoVs with highest 99.5% amino acid identity suggesting possible origin of CaKoVs in Thailand.

**Conclusions:** In conclusion, this study was the first to report the detection and genetic characteristics of CaKoVs in domestic dogs in Thailand. CaKoVs could be detected in both sick and healthy dogs. The virus is frequently detected in younger dogs. Thai CaKoVs were genetically closely related and grouped with Chinese CaKoVs. Our result raises the concerns to vet practitioners that diarrhea in dogs due to canine Kobuvirus infection should not be ignored.

**Keywords:** Canine, Characterization, Detection, Kobuvirus, Thailand

## Background

Kobuvirus (KoV) is a single-strand positive-sense RNA virus. KoV belongs to the family Picornaviridae, genus Kobuvirus, which consists of four species Aichivirus A, B, C and D [1–3]. KoV has been reported in feces of several mammal species including humans, ruminants, pigs, dogs, cats, bats and rodents [3–10]. The Kobuvirus species Aichivirus A contains four types including Aichi virus 1, canine Kobuvirus 1 (CaKoV), Feline Kobuvirus 1 (FeKoV)

and Murine Kobuvirus 1 (MuKoV). Canine Kobuvirus 1 (CaKoV) was first reported in dogs with acute gastroenteritis in the US in 2011 [5, 11]. CaKoV was subsequently reported in dogs in UK, Italy, Australia, Japan, Korea and China [4, 12–15]. The virus was reported in wild carnivores (Jackal and Hyena) and domestic dogs in Tanzania, Africa [16], in foxes in Spain [17] and in foxes [18] and wolves in Italy [19]. Several studies have reported the detection of CaKoV infection in dogs with or without diarrhea and sometime systemic infection [20]. To date, only 12 completed CaKoV genomes are available in the GenBank database.

During September 2016 to September 2018, the center of excellence for emerging and re-emerging infectious diseases in animals (CUEIDAs), Chulalongkorn University conducted

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a survey of canine Kobuvirus in domestic dogs at small animal hospitals in 5 provinces of Thailand. The survey was conducted under the Chulalongkorn University's animal use and care protocol # 1731074. The result of this study provided the first detection and genetic characterization of CaKoV isolated from domestic dogs in Thailand.

## Results

### Canine Kobuviruses in domestic dogs in Thailand

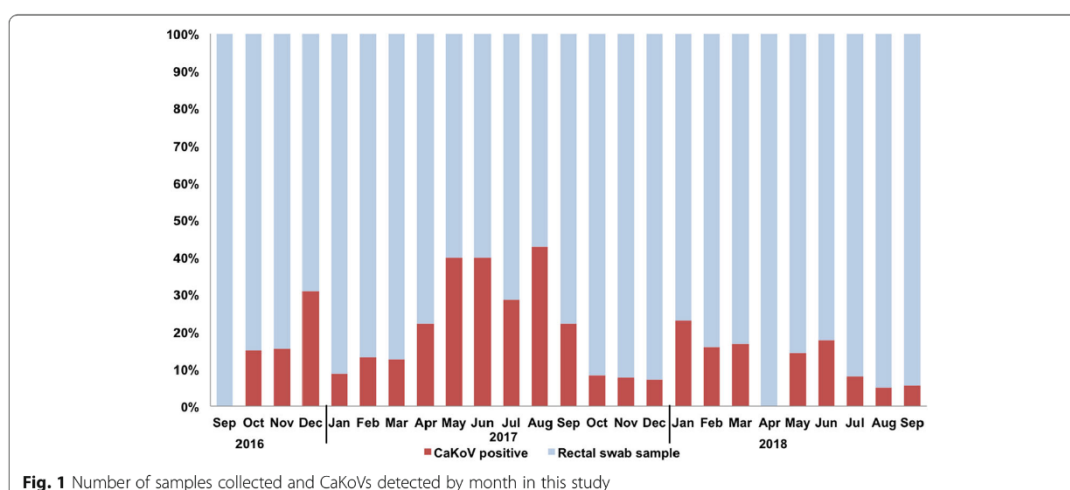
During September 2016 to September 2018, we conducted a survey of viral enteric diseases in domestic dogs in small animal hospitals in 5 provinces of Thailand (Bangkok, Nakhon Ratchasima, Ratchaburi, Suphanburi, and Tak). We tested 307 rectal swab samples for CaKoV by using one-step RT-PCR specific to 3D gene. Based on a two-year survey, we found CaKoV positivity at 17.59% (54/307). CaKoVs could be detected in both sick (19.44% (49/252)) and healthy (9.09% (5/55)) animals. Our result showed no seasonal pattern of CaKoV infection in dogs (Figs. 1 and 2). In relation to age group, CaKoV could be frequently detected in younger dogs at 25.45% (42/165) (Additional file 2: Table S2). The co-infections of CaKoV with other enteric viral pathogens were observed including CaKoV/Canine parvovirus/Canine Coronavirus ( $n = 6$ ), CaKoV/Canine parvovirus ( $n = 20$ ) and CaKoV/Canine Coronavirus ( $n = 2$ ). In this study, 12 CaKoVs were selected and characterized by whole genome sequencing ( $n = 4$ ) or 3D and VP1 gene sequencing ( $n = 8$ ). The viruses were selected to represent epidemiological and demographic data such as age, date of isolation and breed. In this study, nucleotide sequences of the CaKoV were submitted to the GenBank database under the accession numbers MK201776 - MK201795 (Table 1).

### Phylogeny of the Thai canine Kobuviruses

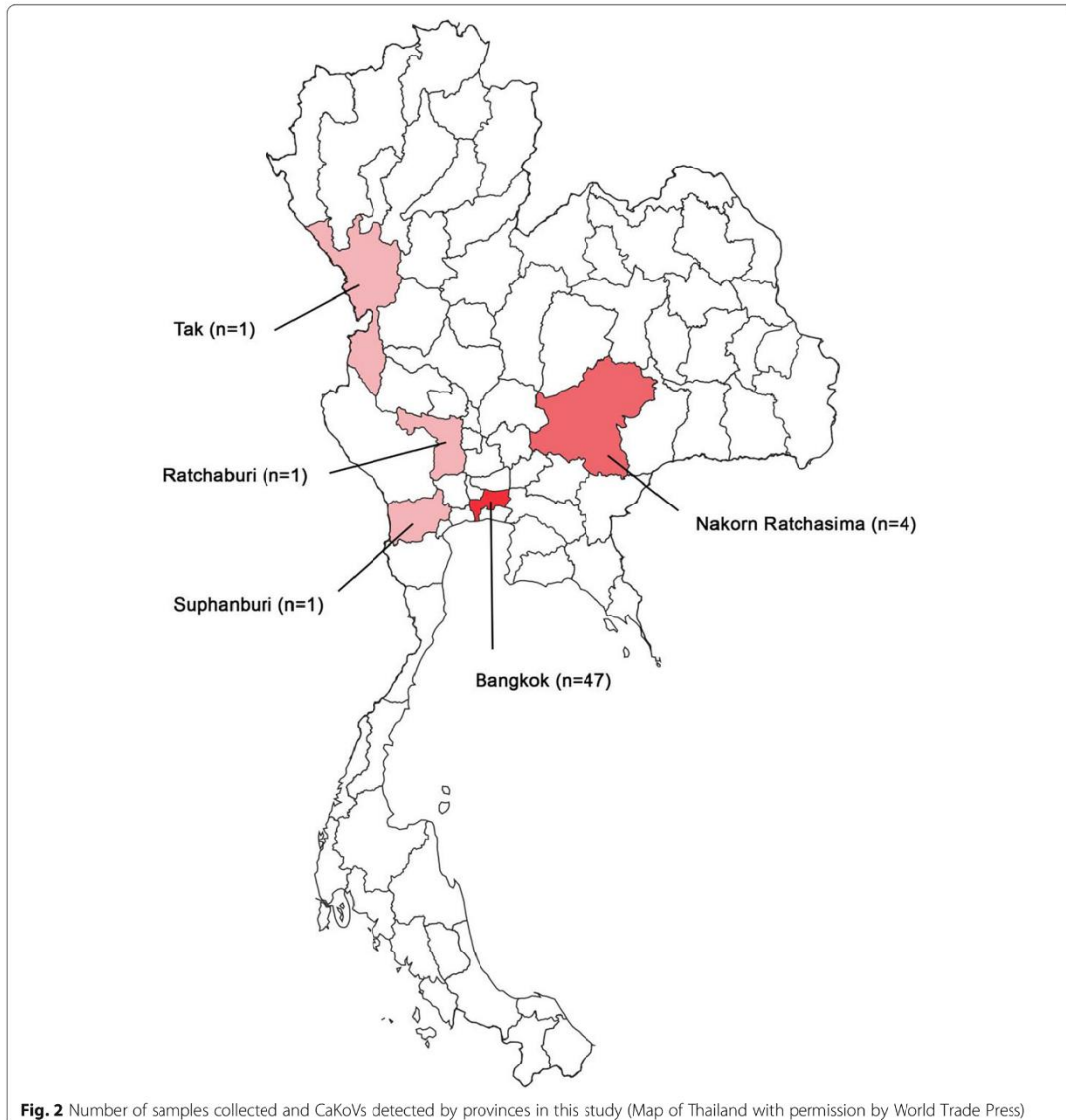
Phylogenetic analysis of whole genome of CaKoVs showed that the Thai CaKoVs were closely related to each other and clustered with Aichivirus A. The cluster Aichivirus A contains Kobuviruses from dogs, cats, rodents, bats and human. While Aichivirus B and C contain Kobuviruses from cattle and pigs, respectively. Based on whole genome sequence, Thai CaKoVs were closely related to Chinese CaKoVs sub-cluster but in separated sub-cluster from the viruses from the US, UK, Brazil and Tanzania (Fig. 3). Phylogenetic analysis of 3D and VP1 of Thai CaKoVs and reference CaKoVs from various animal species were also performed. Similarly, 3D gene of Thai CaKoVs were grouped together with Chinese CaKoVs (G1 sub-cluster) but separated from the viruses in sub-clusters G2 as well as G3 (Fig. 4). Phylogenetic analysis of VP1 gene, the viruses can be clustered into 2 major subgroups, US/EU/Africa subgroup and China/Thailand subgroup (Fig. 5).

### Genetic analysis of the Thai canine Kobuviruses

We compared the nucleotide and deduced amino acid sequences of Thai CaKoVs against those of reference viruses from the US, UK, Italy, China, and Korea (Tables 2 and 3). Our results showed that whole genome of 4 Thai CaKoVs (CU-53, CU-101, CU-249 and CU-716) shared 96.7–99.3% nucleotide similarity (99.6–100% amino acid similarity) to each other and posed highest nucleotide similarity to Chinese CaKoVs including SMCD-59 (97.0% nt and 99.5% aa identity) and CH-1 (96.8% nt and 98.7% aa identity). Our analysis showed that the VP1 protein was the most diverse gene with 93.4–99.9% nucleotide similarity (96.9–100% aa similarity) among Thai CaKoVs and 82.2–96.8% with other reference CaKoVs. The



**Fig. 1** Number of samples collected and CaKoVs detected by month in this study



most variable region of VP1 is position 201–243, especially proline rich region. Putative proline rich region at VP1–228–240 (P<sub>228</sub>XPPPPXPPXP<sub>240</sub>) was also observed in Thai CaKoVs as well as reference viruses (Table 4). In this study, unique amino acids were found in Thai and Chinese CaKoVs at the position, 65 V, 67D, 119L, 138T, 150P, 151M, 153D, 201S, 204Q, 205Q, 201Q, 213T and 241E (Table 4). Analysis of predicted amino acid cleavage sites of

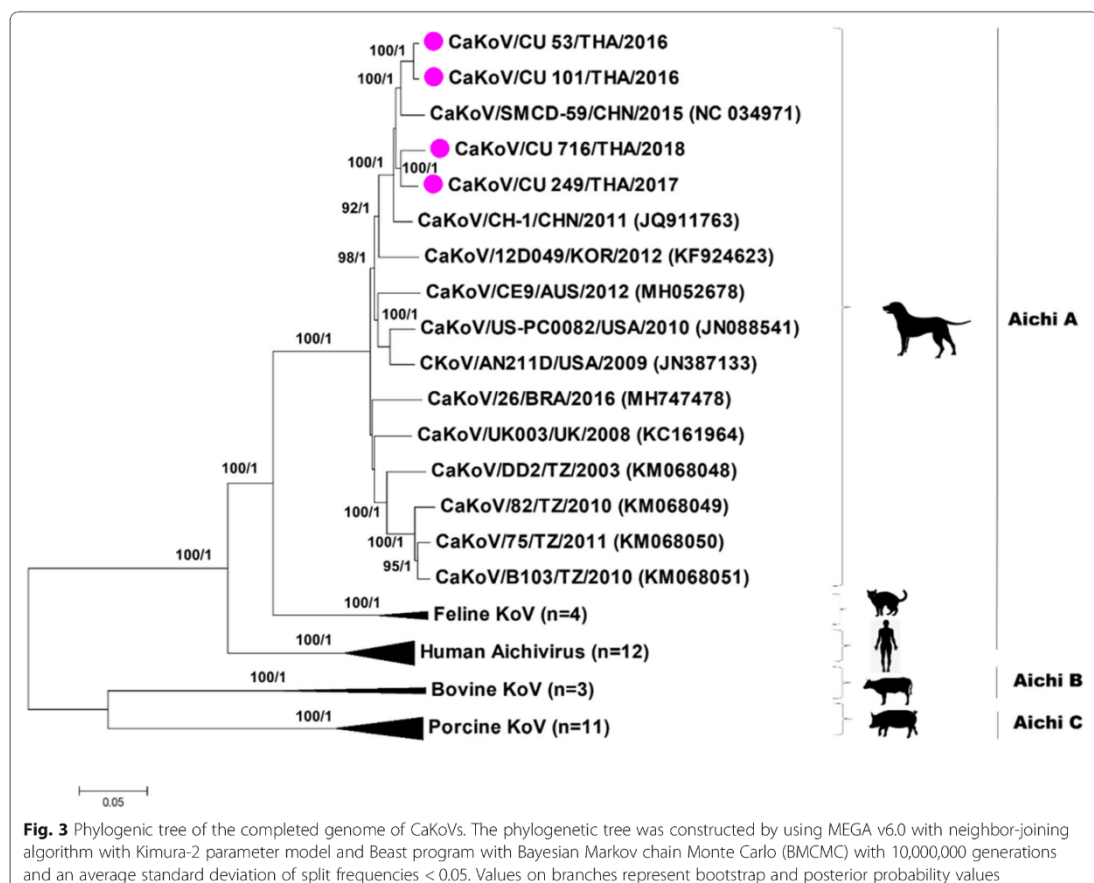
whole genome were conserved among Thai CaKoVs (Table 5).

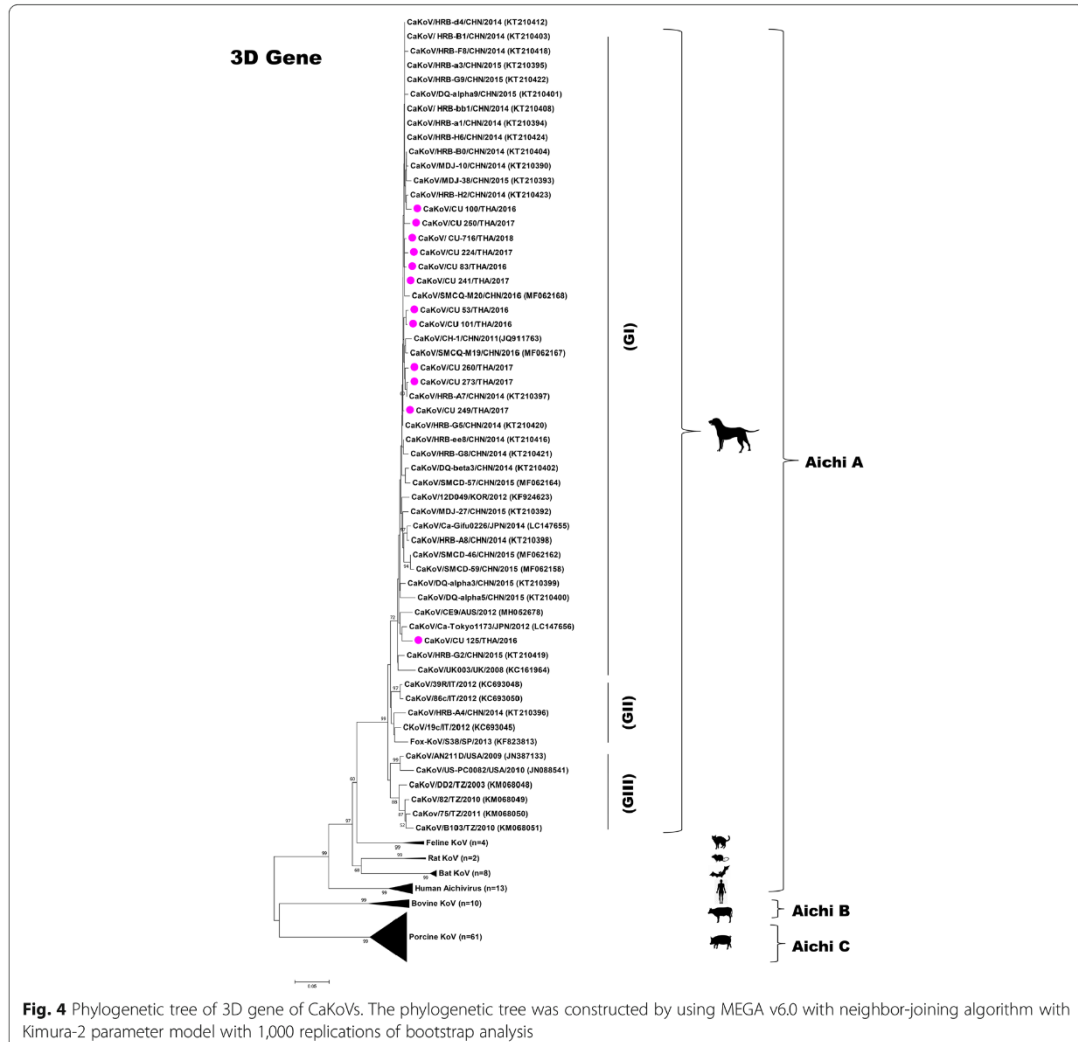
### Discussions

Canine Kobuvirus (CaKoV) is an emerging pathogen in Thailand. To the best of our knowledge, the CaKoV was described in Asia in retrospective study in Korea in 2011 and have been reported in Japan, China and Australia, respectively [2, 15, 17, 21]. However, the CaKoV have

**Table 1** Detail description of Thai CaKoVs characterized in this study

Virus	Date	Location	Region	Age	Breed	Clinical signs	Sequencing	GenBank Accession number
CU-53	Oct-16	Bangkok	Central	2 months	Pomeranian	Diarrhea	WG <sup>a</sup>	MK201776
CU-101	Dec-16	Bangkok	Central	3 months	Pekingese	Diarrhea	WG	MK201777
CU-249	May-17	Bangkok	Central	3 months	Pomeranian	Diarrhea	WG	MK201778
CU-716	Jan-18	Bangkok	Central	12 years	Shizu	Diarrhea	WG	MK201779
CU-83	Nov-16	Bangkok	Central	2 months	Pomeranian	Diarrhea	3D, VP1 <sup>b</sup>	MK201780, MK201788
CU-100	Dec-16	Ratchaburi	Central	6 months	Great Dane	Diarrhea	3D, VP1	MK201781, MK201789
CU-125	Jan-17	Tak	Northern	2 months	Bang Keaw	Asymptomatic	3D, VP1	MK201782, MK201790
CU-224	Feb-17	Bangkok	Central	9 years	Pomeranian	Diarrhea	3D, VP1	MK201783, MK201791
CU-241	Apr-17	Bangkok	Central	3 months	Mixed	Diarrhea	3D, VP1	MK201784, MK201792
CU-250	May-17	Bangkok	Central	3 months	Pomeranian	Diarrhea	3D, VP1	MK201785, MK201793
CU-260	Jun-17	Nakhon Ratchasima	North- Eastern	2 months	German Shepherd	Diarrhea	3D, VP1	MK201786, MK201794
CU-273	Aug-17	Bangkok	Central	2 months	Pomeranian	Diarrhea	3D, VP1	MK201787, MK201795

<sup>a</sup>WG Whole genome sequencing<sup>b</sup>3D, VP1: 3D and VP1 gene sequencing



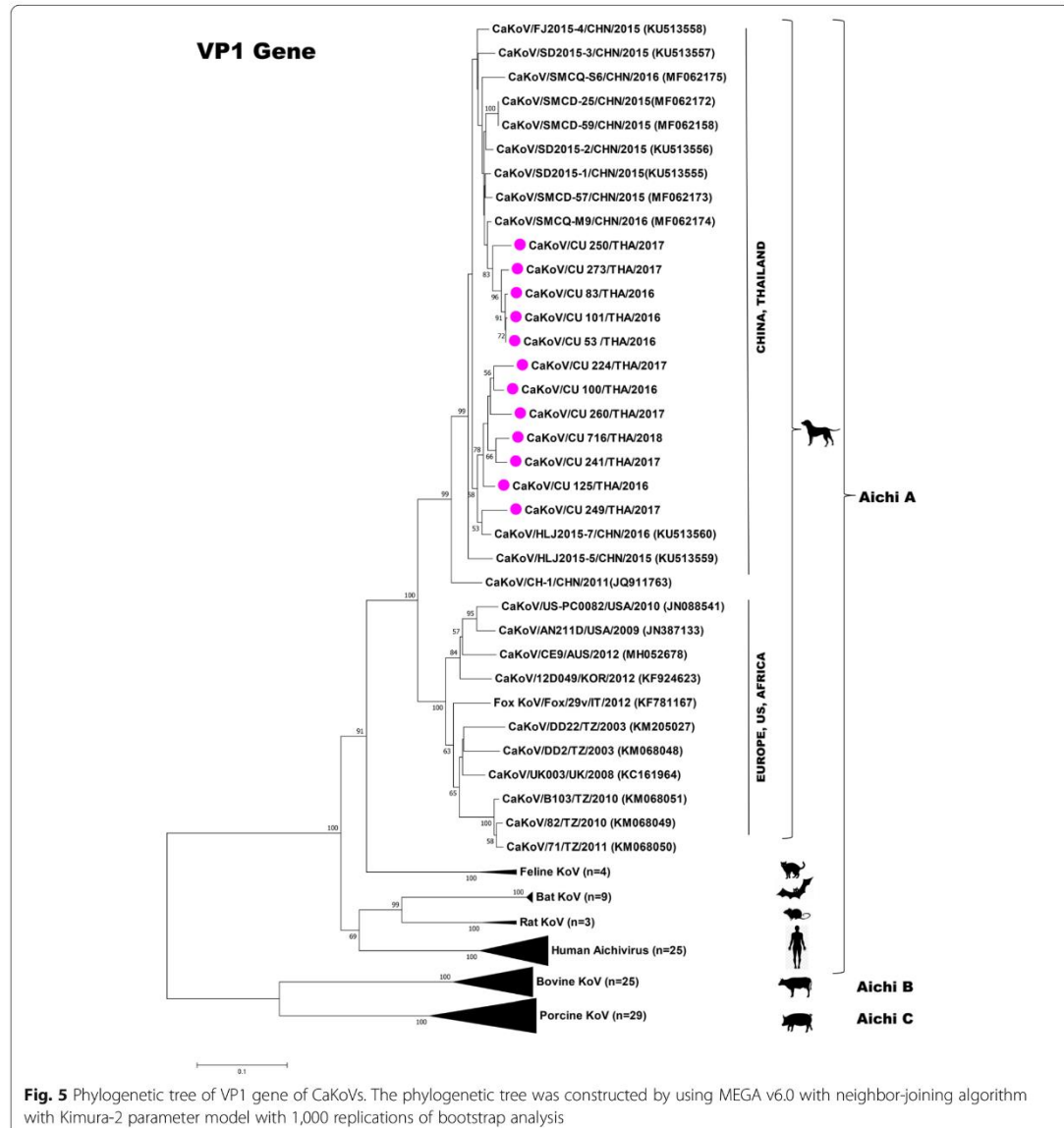
**Fig. 4** Phylogenetic tree of 3D gene of CaKoVs. The phylogenetic tree was constructed by using MEGA v6.0 with neighbor-joining algorithm with Kimura-2 parameter model with 1,000 replications of bootstrap analysis

never been reported in the country or South East Asia region. In this study, during the 2 year-survey program, we found CaKoV positivity at 17.59% in both sick (19.44%) and healthy (9.09%) animals. Compare to other studies, CaKoV % positivity in this study was lower than those in China (54%) and Korea (32.2%) [14, 22]. Our result showed that the CaKoV could be frequently detected in younger dogs at 27% which consistency with previous reports [15]. Similar to other previous studies, co-infections with other enteric viral pathogens were observed such as CaKoV/Canine parvovirus and CaKoV/Canine Coronavirus [12, 14, 15]. Moreover, CaKoVs were detected in both diarrheic and non-diarrheic dogs which

consistent with other studies [2, 15]. Our result supported that this virus may not be the only cause of enteric disease in dogs. Nevertheless, the CaKoV infection have still been identified in symptomatic dogs without other enteric pathogen infections [12]. Our observation supported that the role of CaKoV as a primary pathogen of acute gastroenteritis remain unclear.

In this study, the genome size of 4 Thai CaKoVs is 7,530 bp with one ORF encoding 2,444 amino acids of a putative polyprotein, which comparable to previous reports. Genome organization of CaKoV includes leader protein (L), structural proteins (VP0, VP3, VP1), non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, 3D).





Phylogenetic analyses showed that the Thai CaKoVs were closely related to each other and clustered with Aichivirus A. It is noted that Thai CaKoVs were closely related to Chinese CaKoVs sub-cluster but in separated sub-cluster from the viruses from the US, UK, Brazil and Tanzania (Fig. 3). Phylogenetic analyses of 3D gene showed similar result which Thai CaKoVs were grouped together with Chinese CaKoVs (G1 sub-cluster). This observation regarding to the sub-clusters of CaKoVs was in agreement with the previous study [23]. On the other

hand, based on VP1 gene, the viruses can be clustered into 2 major subgroups, US/EU/Africa subgroup and China/Thailand subgroup which similar to the previous reports [16, 22] (Figs. 4 and 5).

Genetic analyses of Thai CaKoVs showed that whole genome of 4 Thai CaKoVs posed highest nucleotide similarity to Chinese CaKoVs including SMCQ-59 and CH-1. This observation supported phylogenetic analysis that Thai CaKoVs were closely related to Chinese CaKoVs sub-cluster but in separated sub-cluster from

**Table 2** Pairwise comparison of whole genome of Thai Cakovs (CU-101) and reference Cakovs

Virus	Accession number	Year	Country	% nucleotide identity (% amino acid identity)											
				WG5	VP0	VP3	VP1	2A	2B	2C	3A	3B	3C	3D	
Cakov/CU-101/THA/2016	This study	2016	Thailand	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)
Cakov/CU-53/THA/2016	This study	2016	Thailand	99.3 (100)	99.8 (100)	99.3 (100)	99.5 (100)	100 (100)	97.1 (100)	99.4 (100)	100 (100)	100 (100)	100 (100)	100 (100)	98.8 (100)
Cakov/CU-249/THA/2017	This study	2017	Thailand	96.7 (99.6)	96.2 (100)	97.2 (99.6)	94.3 (98.2)	100 (100)	97 (100)	97.2 (100)	97.2 (100)	97.2 (98.9)	96.3 (100)	97.6 (99.7)	97.5 (100)
Cakov/CU-716 /THA/2018	This study	2018	Thailand	96.7 (99.8)	95.2 (100)	97.5 (99.6)	95.2 (99.6)	100 (100)	96 (100)	96.8 (100)	96.8 (100)	96.8 (98.9)	96.3 (96.3)	98.3 (99.7)	97.5 (100)
Cakov/CH-1/CHN/2011	JQ911763	2011	China	96.8 (98.7)	97.4 (99.7)	97.6 (99.6)	91.2 (91.8)	98.3 (100)	97.1 (100)	97.3 (99.7)	98.6 (100)	100 (100)	98.5 (99.7)	97.1 (100)	
Cakov/SMCD-59/CHN/2015	NC034971	2015	China	97 (99.5)	92.5 (98.7)	93 (99.6)	96.5 (97.8)	100 (100)	95.3 (100)	96 (100)	94.7 (97.9)	89 (96.3)	95.6 (99.2)	95.3 (99.6)	
Cakov/12D049/KOR/2012	KF924623	2012	Korea	94.2 (97.9)	93.2 (98.7)	93.2 (99.6)	85.5 (90)	100 (100)	97.3 (100)	96.4 (99.1)	97.2 (98.9)	93.9 (96.3)	96.9 (98.7)	94.7 (98.5)	
Cakov/UNK003/UK/2008	KC161964	2008	UK	93.6 (98.1)	92.4 (99)	95.2 (99.6)	86 (89.6)	100 (100)	93.4 (99)	95.3 (99.7)	94.3 (98.9)	91.5 (96.3)	95.9 (99.2)	96.4 (100)	
Cakov/26/BRA/2016	MH747478	2016	Brazil	92.8 (97.9)	91.1 (99)	91.7 (99.1)	83.2 (86)	100 (100)	95.6 (100)	95.8 (100)	96.5 (98.9)	87.8 (96.3)	94.7 (98.2)	96.5 (99.6)	
Cakov/US-PC0082/USA/2010	JN088541	2010	USA	93.4 (97.7)	91.1 (97.4)	92.3 (99.1)	85.7 (88.2)	100 (100)	93.3 (98)	94.2 (99.4)	92.9 (98.9)	89 (96.3)	94.6 (99)	94.4 (98.9)	
Cakov/CE9/AUS/2012	MH052678	2012	Australia	93.7 (97.6)	97.6 (99.5)	96.4 (100)	85.7 (90)	100 (100)	97.6 (100)	97.6 (100)	96.8 (98.9)	95.1 (100)	97.4 (99.5)	97 (100)	
Cakov/75/TZ/2011	KM068050	2011	African	92.1 (97.5)	92.5 (99.2)	92.4 (99.6)	84.2 (88.5)	100 (100)	96.3 (99.5)	95.6 (99.4)	95 (98.9)	90.2 (96.3)	96.4 (99.2)	96.5 (99.6)	
Cakov/B103/TZ/2010	KM068051	2010	African	92.2 (97.5)	90.8 (96.9)	91.8 (98.7)	84.7 (89.2)	100 (100)	93.4 (99)	93.8 (99.4)	92.9 (98.9)	89 (96.3)	94.6 (99.2)	94.9 (99.3)	
Cakov/DD2/TZ/2003	KM068048	2003	African	92.3 (97.9)	91 (98.7)	94.1 (99.6)	84.3 (89.2)	100 (100)	93.4 (99)	93.2 (98.8)	92.6 (98.9)	89 (96.3)	94.9 (99.2)	94.9 (99.6)	
Cakov/82/TZ/2010	KM068049	2010	African	91.8 (96.5)	91 (97.1)	92.4 (99.1)	84.2 (87.8)	100 (100)	93.3 (98)	92.8 (98.8)	92.2 (97.9)	91.5 (96.3)	94.4 (98.7)	94.4 (98.9)	



**Table 3** Pairwise comparison of 3D and VP1 genes of Thai CaKoVs (CU-101) and reference CaKoVs

Viruses	Accession number	Year	Country	% nucleotide identity (% amino acid identity)	
				3D	VP1
CaKoV/CU-101/THA/2016	This study	2016	Thailand	100 (100)	100 (100)
CaKoV/CU-53/THA/2016	This study	2016	Thailand	99.5 (100)	99.9 (100)
CaKoV/CU-83/THA/2016	This study	2016	Thailand	98.8 (100)	99.7 (100)
CaKoV/CU-100/THA/2016	This study	2016	Thailand	97.9 (100)	93.6 (97.8)
CaKoV/CU-125/THA/2016	This study	2016	Thailand	97.1 (98.6)	94.9 (97.8)
CaKoV/CU-224/THA/2017	This study	2017	Thailand	98.6 (100)	93.6 (97.8)
CaKoV/CU-241/THA/2017	This study	2017	Thailand	99.0 (100)	94.5 (98.7)
CaKoV/CU-249/THA/2017	This study	2017	Thailand	98.8 (100)	93.6 (97.4)
CaKoV/CU-250/THA/2017	This study	2017	Thailand	98.1 (100)	96.6 (96.9)
CaKoV/CU-260/THA/2017	This study	2017	Thailand	98.6 (100)	93.4 (96.9)
CaKoV/CU-273/THA/2017	This study	2017	Thailand	98.6 (100)	98.5 (99.1)
CaKoV/CU-716/THA/2018	This study	2018	Thailand	98.8 (100)	94.3 (98.7)
CaKoV/26/BRA/2016	MH747478	2016	Brazil	97.1 (99.3)	82.2 (84.2)
CaKoV/CE9/AUS/2012	MH052678	2012	Australia	97.6 (100)	83.7 (87.7)
CaKoV/B103/TZ/2010	KM068051	2010	African	93.6 (98.6)	84.8 (88.2)
CaKov/75/TZ/2011	KM068050	2011	African	94.0 (97.9)	83.8 (86.4)
CaKoV/82/TZ/2010	KM068049	2010	African	94.5 (98.6)	84.3 (86.4)
CaKoV/DD2/TZ/2003	KM068048	2003	African	94.8 (99.3)	84.0 (87.7)
CaKoV/UK003/UK/2008	KC161964	2008	UK	96.0 (100)	85.3 (88.2)
CaKoV/US-PC0082/USA/2010	JN088541	2010	USA	94.0 (99.3)	84.5 (86.4)
CaKoV/AN211D/USA/2009	JN387133	2009	USA	95.2 (99.3)	84.4 (86.8)
CaKoV/86c/IT/2012	KC693050	2012	Italy	96.0 (99.3)	N/A
CKoV/19c/IT/2012	KC693045	2012	Italy	96.2 (99.3)	N/A
CaKoV/Ca-Gifu0226/JPN/2014	LC147655	2014	Japan	97.6 (99.3)	N/A
CaKoV/Ca-Tokyo1173/JPN/2012	LC147656	2012	Japan	97.9 (100)	N/A
CaKoV/12D049/KOR/2012	KF924623	2012	Korea	97.1 (100)	84.7 (89.0)
CaKoV/CH-1/CHN/2011	JQ911763	2016	China	97.9 (100)	91.3 (89.9)
CaKoV/SMCD-59/CHN/2015	MF062158	2015	China	97.1 (100)	96.4 (96.9)
CaKoV/SMCD-57/CHN/2015	MF062173	2015	China	97.9 (100)	96.8 (97.8)

the viruses from the US, UK, Brazil and Tanzania. Of all viral genes, the VP1 gene was the most diverse gene among Thai CaKoVs and other reference CaKoVs. Similar observation was also reported in previous study that VP1 protein is the most variable capsid protein [24]. It is noted that the putative proline rich region at VP1-228-240 (P<sub>228</sub>-XPPPPXPPXP<sub>240</sub>) was observed both in Thai CaKoVs and reference viruses. Previous studies indicated that proline rich region may associate with enteric receptor binding of the viruses [14, 24]. It is noted that Thai CaKoVs posed unique PPP (VP1; 228-240), which also observed most reference viruses from China, Korea, Japan, US, UK suggesting unique characteristic. These unique amino acids were not observed in the CaKoV from the Australia (CE9), Brazil (BRA/26) and Tanzania (TZ/75, TZ82) [16, 20]. However, the association

of these unique amino acids and viral pathogenesis is still need to be further investigated. Based on genetic analysis, unique amino acids at the position, 65 V, 67D, 119L, 138 T, 150P, 151M, 153D, 201S, 204Q, 205Q, 201Q, 213 T and 241E were observed. These unique amino acids of China/Thailand sub-cluster could be benefit for the detection of virus origin or diagnostic purpose in the future. Similar to previous study, analysis of predicted amino acid cleavage sites of whole genome were conserved among CaKoVs except one variation at 776/777 (VP3/VP1) which unique in wild carnivores [16].

### Conclusions

In conclusion, this study is the first to report of canine Kobuvirus in dogs in Thailand. CaKoVs were mostly detected in clinical dogs of young age. However, the viruses

**Table 4** Genetic analysis of Thai CaKoVs compared with reference CaKoVs at proline rich region

Viruses	Accession number	Year	Country	Amino acid at position													Proline rich region (228–240)
				65	67	119	138	150	151	153	201	204	205	210	213	241	
CaKoV/CU-101/THA/2016	This study	2016	Thailand	V	D	L	T	P	M	D	S	Q	Q	Q	T	E	PRAPPLPLPTP
CaKoV/CU-53/THA/2016	This study	2016	Thailand	V	D	L	T	P	M	D	S	Q	Q	Q	T	E	PRAPPLPLPTP
CaKoV/CU-249/THA/2017	This study	2017	Thailand	V	D	L	T	P	M	D	S	Q	Q	Q	T	E	PRAPPLPLPTP
CaKoV/CU-716/THA/2018	This study	2018	Thailand	V	D	L	T	P	M	D	S	Q	Q	Q	T	E	PRAPPLPLPTP
CaKoV/SMCQ-M9/CHN/2016	MF062174	2016	China	V	D	L	T	P	M	D	S	Q	Q	Q	T	E	PRAPPLPLPTP
CaKoV/SMCD-59/CHN/2015	NC 034971	2015	China	V	D	L	T	P	M	D	S	Q	Q	Q	T	E	PRAPPLPLPTP
CaKoV/12D049/KOR/2012	KF924623	2012	Korea	L	N	V	M	S	E	N	T	V	E	S	S	A	PRAPPLPLPTP
CaKoV/CE9/AUS/2012	MH052678	2012	Australia	L	N	V	M	S	E	N	T	V	E	S	S	T	PRAPP_LPLPTP
CaKoV/AN211D/USA/2009	JN387133	2009	USA	L	N	P	M	S	E	N	T	V	E	S	S	A	PRAPPLPLPTP
CaKoV/US-PC0082/USA/2010	JN088541	2010	USA	L	N	V	M	S	E	N	T	V	E	S	S	A	CPVPPPLPLPTP
CaKoV/UK003/UK/2008	KC161964	2008	UK	L	N	V	M	S	E	N	T	V	E	S	S	T	PRAPPLPLPTP
CaKoV/26/BRA/2016	MH747478	2016	Brazil	L	N	V	M	S	E	N	T	V	E	S	S	T	HGAPPLPLPTP
CaKoV/75/TZ/2011	KM068050	2011	Africa	L	N	V	M	S	E	N	T	A	E	S	S	T	CPVPPPLPLPTP
CaKoV/82/TZ/2010	KM068049	2010	Africa	L	N	V	M	S	E	N	T	A	E	S	S	T	CPVPPPLPLPTP
CaKoV/B103/TZ/2010	KM068051	2010	Africa	L	N	V	M	S	E	N	T	A	E	S	S	T	PRAPPLPLPTP
CaKoV/DD2/TZ/2003	KM068048	2003	Africa	L	N	V	M	S	E	N	T	V	E	S	S	T	PRAPPLPLPTP

could be detected from both healthy and sicked dogs. Genetic and phylogenetic analyses showed that whole genomes of Thai CaKoVs were closely related to Chinese CaKoVs in 2015 (SMCD-59) with high nucleotide similarity suggesting a possible origin of CaKoVs in Thailand. CaKoV is considered as an emerging viral pathogen in the domestic dogs. Since CaKoVs have

never been reported in the country and SEA region, the detection and characterization of CaKoV from different parts of the regions should be extended for better understanding the epidemiology and evolution of CaKoVs. Our result raises the concerns to vet practitioners that diarrhea in dogs due to canine Kobuvirus infection should not be ignored.

**Table 5** Genetic analysis of Thai CaKoVs compared with reference CaKoVs at putative amino acid cleavage sites

Viruses	Year	Country	Amino acid position									
			171/172	553/554	776/777	1054/1055	1165/1166	1330/1331	1665/1666	1759/1760	1786/1787	2176/2177
CU-53	2016	Thailand	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G
CU-101	2016	Thailand	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G
CU-249	2017	Thailand	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G
CU-716	2018	Thailand	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G
12D049	2012	Korea	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G
UK003	2008	UK	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G
26/BRA	2016	Brazil	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G
SMCD-59	2015	China	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G
CE9	2012	Australia	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G
B103	2010	Africa	Q/G	Q/H	Q/T <sup>a</sup>	Y/V	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G
75	2011	Africa	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G
82	2010	Africa	Q/G	Q/H	Q/T <sup>a</sup>	Y/V	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G
DD2	2003	Africa	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G
US-PC0082	2010	USA	Q/G	Q/H	Q/A	Y/V	Q/G	Q/G	Q/G	Q/A	Q/G	Q/G

<sup>a</sup>Q/T unique cleavage site (only found in Africa isolates)

## Methods

### Sample collection

Sample collection was conducted in domestic dogs at small animal hospitals in Bangkok and vicinity of Thailand During September 2016 to September 2018. 307 rectal swab samples were collected from healthy dogs ( $n = 55$ ) and dogs with gastroenteritis symptoms ( $n = 252$ ) including vomiting, watery diarrhea, hemorrhagic diarrhea and dehydration. The swab samples were collected from dogs of young age (< 1 year) ( $n = 165$ ), adult (1–5 years) ( $n = 98$ ) and older (> 5 years) ( $n = 44$ ). The animal demographic data including age, sex, breed, and vaccination history were also recorded. The ethics was conducted under the Chulalongkorn University's animal use and care protocol # 1731074. The consent to participate of the owners of the animals used in this study was obtained in writing.

### Canine Kobuvirus (CaKoV) detection

All 307 samples were subjected to canine Kobuvirus identification by one step RT-PCR using primers specific to 3D gene of CaKoV [21]. First, RNA extraction was performed using the QIASymphony DSP Viral/Pathogen mini kit (Qiagen, Hilden, Germany) following manufacturer's instructions. To detect CaKoV, RNA samples were screened for 3D gene of CaKoV by using one step RT-PCR assay. The primers used in this study were previously described including UIF (5'-CATGCTCCTCGGTGGTCTCA-3') and U1R (5'-GTCCGGGTCCATCACAGGGT-3') [21]. Briefly, one-step RT-PCR was conducted in a total final volume of 25  $\mu$ l comprising 3  $\mu$ l of template RNA, 15  $\mu$ l of 2xReaction Mix (Invitrogen, USA), 0.6  $\mu$ l of 10  $\mu$ M forward and reverse primers, 1.2  $\mu$ l of SuperScript III RT (Invitrogen, USA) and distilled water to final volume 25  $\mu$ l. The condition of RT-PCR assay included cDNA synthesis step at 55  $^{\circ}$ C for 30 min, next to an initial denaturation step at 94  $^{\circ}$ C for 2 min, following 40 cycles of denaturation at 94  $^{\circ}$ C for 30 s, annealing at 52  $^{\circ}$ C for 30 s and extension at 68  $^{\circ}$ C for 1 min, as well as, final extension step at 68  $^{\circ}$ C for 5 min. To confirm CaKoV, 4  $\mu$ l of PCR products were run on a 1.5% agarose gel, which mixed with Red Safe at 100 V for 45 min. The expected size of CaKoV positive amplified products was 631 bp. Due to dogs showed clinical signs similar to other canine viral enteric diseases, all samples were also tested for Canine Parvovirus ( $n = 307$ ), Canine Rotavirus ( $n = 307$ ) and Canine Coronavirus ( $n = 30$ ) [25–27].

### Canine Kobuvirus characterization

In this study, four CaKoV positive samples (CU-53, CU-101, CU-247 and CU-716) were selected for whole genome sequencing and additional eight CaKoV positive samples were selected for 3D and VP1 gene sequencing. The CaKoVs were selected based on epidemiological and demographic data such as age, date of isolation, breed,

and vaccination history. For sequencing, nucleotide sequences of each gene of the viruses were amplified by new primer sets designed by using Primer 3 plus program [28]. List of oligonucleotide primers is provided in Additional file 1: Table S1 In brief, PCR was proceed in a final volume of 30  $\mu$ l containing 2  $\mu$ l of cDNA, 0.4  $\mu$ M of each forward and reverse primer, 1X TopTaq Master Mix, 1X Coral Load, and distilled water. The PCR condition was set as initial denaturation at 94  $^{\circ}$ C for 3 min; 40 cycles of denaturation at 94  $^{\circ}$ C for 30 s, annealing at 50–55  $^{\circ}$ C for 45 s, extension at 72  $^{\circ}$ C for 1–1.30 min; and final extension at 72  $^{\circ}$ C for 7 min. PCR products were then purified and sequenced (1st Base Laboratories Sdn Bhd, Malaysia). Nucleotide sequences were edited, validated and assembled by using SeqMan software v.5.03 (DNASTAR Inc.; Wisconsin, USA).

### Phylogenetic and genetic analyses of canine Kobuviruses

The phylogenetic and genetic analyses were performed by comparing nucleotide sequences of Thai CaKoVs with those of Kobuvirus available from the GenBank database. The reference nucleotide sequences of CaKoVs were retrieved based on their different geographic locations, host species and date of isolation. Phylogenetic analysis of CaKoV was performed by using MEGA v.6.0 (Tempe, AZ, USA) [29] with neighbor-joining method with Kimura 2-parameter with 1,000 bootstrap replicates and Beast program with Bayesian Markov chain Monte Carlo (BMCMC) with 10,000,000 generations and an average standard deviation of split frequencies < 0.05 [30]. For genetic analysis, the nucleotide sequences and deduced amino acids of CaKoV were aligned and compared using MegAlign software v.5.03 (DNASTAR Inc.; Wisconsin, USA). Pairwise comparison of nucleotides and amino acids of Thai CaKoV and those of reference CaKoVs were conducted. The variable and unique amino acids related to receptor binding of the viruses and host preferences of CaKoVs were monitored.

### Additional files

**Additional file 1: Table S1.** Oligonucleotide primers used for CaKoV whole genome sequencing. (DOCX 35 kb)

**Additional file 2: Table S2.** Association of age of CaKoVs detection in this study. (DOCX 34 kb)

### Abbreviations

CaKoV: Canine Kobuvirus; FeKoV: Feline Kobuvirus; KoV: Kobuvirus; MuKoV: Murine Kobuvirus

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**Authors' contributions**

AA supervised and principle investigator of the project. KC, TJ, SC and RT conducted and coordinated the study, sample collection, virus identification and virus characterization. KC, NB, SB conducted data analysis and drafting the manuscript. AA drafting, revising and corresponding the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article and supplement tables.

**Ethics approval and consent to participate**

Ethics and consent to participate in the study was conducted under the Chulalongkorn University's animal use and care protocol (IACUC) # 1731074.

**Consent for publications**

The consent to participate of the owners of the animals used in this study was obtained in writing.

**Competing interests**

All authors in this paper declare that they have no competing interests.

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## APPENDIX C

## Evidence of human norovirus infection in dogs, Thailand

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**Keywords:** Dog, Evidence, Human, Norovirus, Thailand



# Human Norovirus Infection in Dogs, Thailand

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In July 2018, recombinant norovirus GII.Pe-GII.4 Sydney was detected in dogs who had diarrhea in a kennel and in children living on the same premises in Thailand. Whole-genome sequencing and phylogenetic analysis of 4 noroviruses from Thailand showed that the canine norovirus was closely related to human norovirus GII.Pe-GII.4 Sydney, suggesting human-to-canine transmission.

Norovirus infection is a major cause of endemic and epidemic acute gastroenteritis. These viruses have been classified into 7 genogroups on the basis of the major capsid protein, VP1. Noroviruses GI, GII, and GIV can infect humans, GII pigs, GIII and GV ruminants and mice, and GVI and GVII dogs (1). The evolutionary mechanism and typing of noroviruses can be analyzed on the basis of recombination between the genes for RNA-dependent RNA polymerase and VP1 (2). Newly emerged norovirus strains might lead to increasing incidence of infection worldwide (3). The predominant genotype of noroviruses in humans is GII.4. Genetic diversity of noroviruses has been reported in a wide range of animals (e.g., pigs, cattle, and dogs).

In 2007, canine noroviruses in Italy were reported to have the GIV.2 genotype (4). Subsequently, these viruses have been reported to cause diseases in dogs in Asia and Europe (5–8). The seroprevalence of human noroviruses in dogs in the United Kingdom was reported to be 13% (6). The GII.4 genotype (variants GII.4-2006b and GII.4-2008) was reported in dogs in Finland, indicating that human noroviruses could be transmitted to and cause diarrhea in dogs (9). In humans, antibodies against canine norovirus were also reported in veterinarians, who experienced high risk

of exposure (10). However, only a few reports describe human norovirus infections in dogs, and limited numbers of complete genomes of canine noroviruses are available in GenBank. We report evidence of human norovirus infection in dogs from a kennel and children on the same premises in Thailand.

## The Study

On July 27, 2018, we investigated acute gastroenteritis in dogs in a dog kennel. An outbreak occurred in a small-scale dog kennel that contained 18 adult dogs in Suphanburi, central Thailand. Clinical signs in bitches and puppies were fever, acute watery diarrhea, and mild dehydration (Appendix Figure 1, <https://wwwnc.cdc.gov/EID/article/26/2/19-1151-App1.pdf>). Information for the outbreak investigation indicated that 2 weeks earlier (July 18), 2 children (8 months and 2 years of age) who lived on the kennel premises were hospitalized because of vomiting and watery diarrhea. These children recovered within 1 week. During hospitalization, human cases were diagnosed and confirmed as norovirus infection by using a rapid test kit (RIDA QUICK Norovirus, <https://clinical.r-biopharm.com>). Five adults, 2 children, and 18 adult dogs were living on the premises. All dogs were housed in the kennel; only 2 apparently pregnant dogs (CU21939 and CU21952) were moved into the house of the owner. The 2 apparently pregnant dogs were kept in close contact with children.

On August 2, 2018, a pregnant dog gave birth to 6 puppies, and the other bitch was found to have a false pregnancy. During the 6 weeks (July 27–September 5) of the norovirus outbreak, 2 (11.11%) of 18 dogs (the 2 apparently pregnant dogs kept in the house of the owner) and 5 (83.33%) of 6 puppies showed clinical signs of infection (Appendix Table 1). After treatment and hygiene management, including separation of dogs, frequent cleaning, and disinfection, all dogs recovered, and no deaths occurred.

Animal samples were collected and examined at the Center of Excellence for Emerging and

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**Table.** Characteristics of noroviruses from humans and dogs, Thailand, July 2018\*

Virus	Host	Sample	Age	GenBank accession no.
GII/Hu/THA/2018/GII.Pe-GII.4/CU21953	Human	Feces	2 y	MK928496
GII/Hu/THA/2018/GII.Pe-GII.4/CU21954	Human	Feces	8 mo	MK928497
GII/Ca/THA/2018/GII.Pe-GII.4/CU21939	Dog	Rectal swab	2 y	MK928498
GII/Ca/THA/2018/GII.Pe-GII.4/CU21952	Dog	Rectal swab	3 y	MK928499

\*Whole-genome sequences were tested for all isolates.

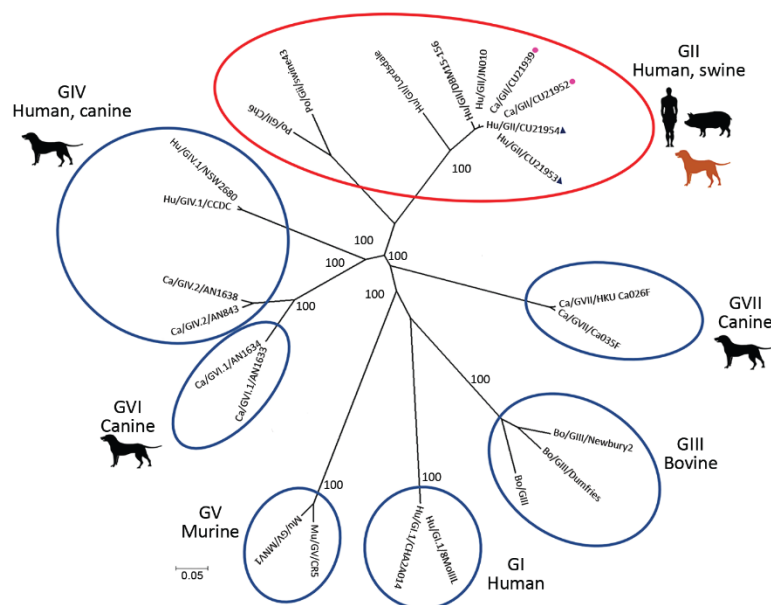
Re-emerging Infectious Diseases in Animals, Chulalongkorn University (Bangkok, Thailand). Studies were approved by the Institutional Animal Care and Use Committee (approval no. 1731074). Human samples were collected and submitted to the Center of Excellence for Clinical Virology under the institutional review board of Chulalongkorn University (Institutional Review Board no. 634/59).

During the 4 visits in the study, we examined 75 samples (4 stool samples from 2 children, 71 rectal swab specimens from 18 adult dogs and 6 puppies). We detected norovirus by using a reverse transcription PCR specific for the RNA-dependent RNA polymerase gene as described (11,12) (Appendix). We detected norovirus in samples from children (4/4), adult dogs (2/53), and puppies (10/18) (Appendix Table 1). All human samples were positive for norovirus at the first (July 27) and third (August 25) visits. The 2 litters with clinical signs were positive for norovirus at the first visit (July 27). Their puppies (5/6) were positive at the second (August 18) and third (August 25) visits. Our findings are consistent with a previous

report that animals can shed noroviruses for a long period (4). All samples were also tested for canine parvovirus type 2, rotavirus A, canine coronavirus, and canine distemper virus to rule out other canine enteric diseases; all showed negative results (Appendix Table 1).

We selected 4 of the noroviruses, 2 from humans (CU21953 and CU21954) and 2 from dogs (CU21939 and CU21952), for whole-genome sequencing by using oligonucleotide primer sets (Appendix). We then submitted nucleotide sequences for these viruses (GenBank accession nos. MK928496–9) (Table). Phylogenetic analysis showed that the noroviruses in this investigation clustered in genotype GII.4. In general, canine noroviruses are commonly grouped into genogroups GIV, GVI, and GVII. In contrast, noroviruses from these dogs were closely related to human noroviruses and viruses in genogroup GII (Figure 1).

Phylogenetic analysis of partial open reading frame 1 (ORF1) and ORF2 showed that all noroviruses from this investigation clustered with norovirus GII.Pe-GII.4 Sydney 2012, which were reported to be



**Figure 1.** Phylogenetic tree of whole-genome sequences of canine noroviruses (red dots) and human noroviruses (blue triangles) from Thailand and reference sequences. Genogroups GI–GVII are indicated by red oval and blue ovals. The tree was constructed by using MEGA version 7.026 (<https://www.megasoftware.net>) with the neighbor-joining algorithm and bootstrap analysis with 1,000 replications. Numbers along branches are bootstrap values. Scale bar indicates nucleotide substitutions per site.

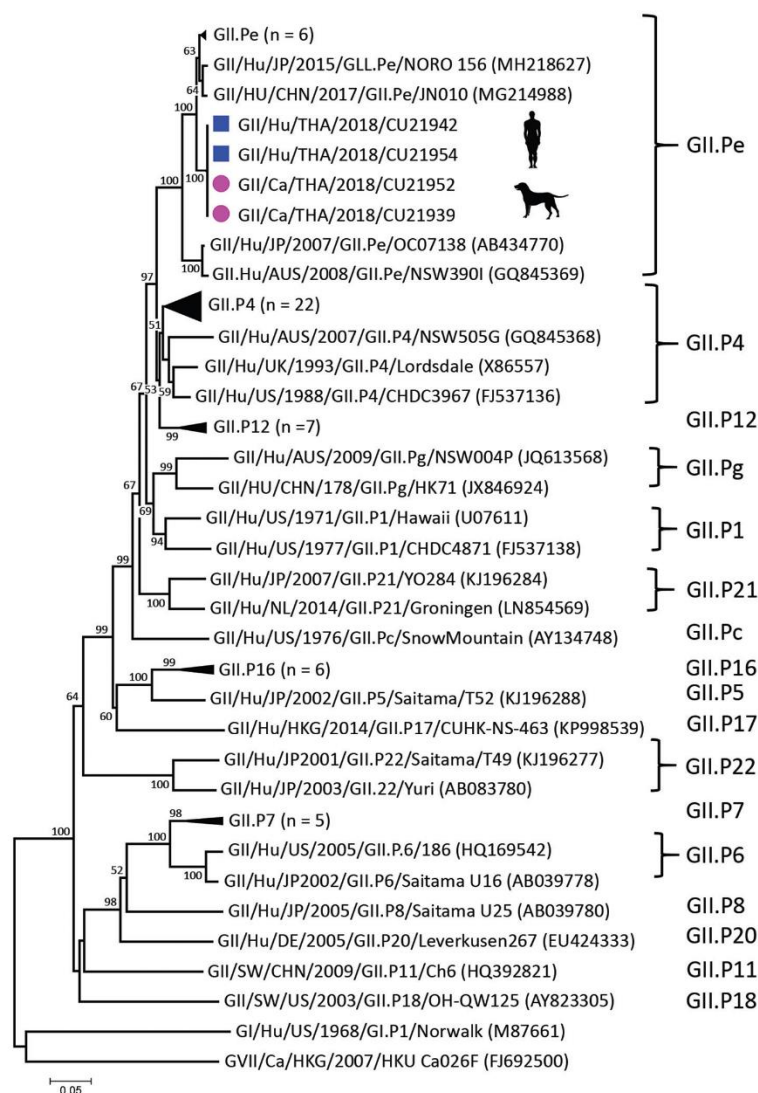
## DISPATCHES

circulating worldwide (Figure 2; Appendix Figure 2) (3). Noroviruses from dogs in this study (GII.4 Sydney) were in different clusters from canine noroviruses 3–09 (GII.4 Den Haag) and 261–10 and 1C-09 (GII.4 unclassified) reported in Finland (9).

We compared nucleotide and deduced amino acids of the noroviruses from this investigation with reference canine and human noroviruses. On the basis of antigenic epitopes (A–E) of major capsid protein that correlate with blockade of neutralization antibodies,

the noroviruses from Thailand had specific amino acids in specific positions consistent with those for human norovirus GII.Pe–GII.4 Sydney, which were not observed in human norovirus genogroups GI and GIV and canine norovirus genogroups GIV and GVII (Appendix Table 2).

Pairwise comparisons of whole-genome sequences showed that the viruses had 99.90% nt identities (only 3 nt differences in ORF2; T1176C [silent mutation 392G], C1354T [silent mutation 452L] and



**Figure 2.** Phylogenetic tree of open reading frame 1 of canine noroviruses (purple dots) and human noroviruses (blue squares) from Thailand and reference sequences. Tree was constructed by using MEGA version 7.026 (<https://www.megasoftware.net>) with the neighbor-joining algorithm and bootstrap analysis with 1,000 replications. Numbers along branches are bootstrap values, and numbers on the right indicate genogroups. Scale bar indicates nucleotide substitutions per site.



in ORF3; T803A [V268E] to each other and highest nucleotide identities to human norovirus from China [99.00%; JN010] and the human norovirus reference Sydney strain [97.6%; NSW0514]). On the basis of partial ORF2 sequences, we showed that the canine noroviruses from this investigation were different from canine noroviruses GII.4 (3-09, 1C-09, and 261-10; 91.6% nt identities) and GIV, GVI, and GVII (52.90%–55.50% nt identities) (Appendix Table 3).

### Conclusions

We report infection of dogs with human norovirus GII.4 Sydney. Human noroviruses have been reported in dogs in Finland (GII.4 Den Haag and GII.4 unclassified) (9). Dogs showed mild clinical signs of acute watery diarrhea, similar to that for human norovirus infection, and low levels of illness and death. Similar observations have also been reported in other studies (8,13). In this study, children had been hospitalized 2 weeks before the investigation. Disease developed in dogs and puppies after they shared the same premises and possible direct contact with the children. This observation suggests potential human-to-dog transmission of human noroviruses. Genetic and phylogenetic analyses confirmed that whole genomes of canine and human noroviruses were closely related to human norovirus GII.Pe-GII.4 Sydney, suggesting that a common strain is circulating in Thailand and worldwide (14,15). However, in our study, it is not clear how and when the viruses were introduced to children and dogs.

In summary, we demonstrated evidence of norovirus GII.Pe-GII.4 infection in humans and dogs in Thailand. Dog owners and veterinarians should pay more attention to norovirus infection as a potential zoonotic and reverse zoonotic disease in households, animal hospitals, and shelters. Expanded surveillance for norovirus is needed to determine its status and distribution in human and dog populations.

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## APPENDIX D

Emergence of canine parvovirus type 2c in domestic dogs and cats from Thailand

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**Keywords:** Canine Parvovirus, Characterization, Detection, Emergence, Thailand

## Emergence of canine parvovirus type 2c in domestic dogs and cats from Thailand

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### Abstract

Canine parvovirus type 2 (CPV-2) is an important pathogen causing haemorrhagic enteritis in domestic dogs and wildlife worldwide. In early 2000, canine parvovirus type 2c (CPV-2c) was first reported and subsequently became a predominant subtype circulating in Europe and the Americas. CPV-2c has also been reported in Asia, including cases in China, India, Taiwan and Vietnam. However, CPV-2c has never been reported in Thailand. In this study, we conducted viral enteric disease surveillance in dogs and cats in Thailand during 2016–2018. During 20 months of surveillance, 507 rectal swab samples were collected from dogs ( $n = 444$ ) and cats ( $n = 63$ ) with and without clinical signs. The samples were examined for parvovirus by using VP2 gene-specific PCR for parvovirus. Our results showed that the positivity of canine parvovirus (CPV) was 29.95% and that of feline parvovirus (FPV) was 58.73%. In this study, we characterized 34 parvoviruses by VP2 gene sequencing. Moreover, two Thai-CPV-2 (Dog/CU-24 and Cat/CU-21) were characterized by whole genome sequencing. The phylogenetic results showed that Thai-CPV-2 had the highest nucleotide identities and clustered with Asian-CPV-2c but were in separate subclusters from the North American and European CPV-2c. Similarly, whole genome analyses showed that Thai-CPVs are closely related to Asian-CPV-2c, with unique amino acids at positions 297A, 324I, 370R and 426E. In summary, our results demonstrated the emergence of Asian-CPV-2c in dogs and cats in Thailand. Thus, the surveillance of CPV-2 in domestic dogs and cats should be further conducted on a larger scale to determine the dynamics of predominant variants and their distributions in the country and in the Southeast Asia region.

### KEYWORDS

canine parvovirus, characterization, detection, emergence, Thailand

## 1 | INTRODUCTION

Canine parvovirus type 2 (CPV-2) is an important pathogen for domestic dogs and wildlife worldwide. CPV-2, a non-enveloped, single-stranded DNA virus, belongs to the family Parvoviridae.

CPV-2 causes acute haemorrhagic enteritis and myocarditis in dogs with high morbidity and frequent mortality (ranging 10%–90%). In 1977, it was first reported that CPV-2 arose from feline panleukopenia virus (FPV) with at least six coding nucleotide differences in the VP2 gene. CPV-2 can be further grouped into

three antigenic variants, including CPV-2a, CPV-2b and CPV-2c, based on unique amino acid residues at the positions 297 and 426 of VP2 (Buonavoglia et al., 2001). CPV-2a and CPV-2b were reported in 1979 and 1984, with unique amino acid residues as 426N and 426D, respectively. Both CPV-2a and CPV-2b variants are distributed worldwide and infect both dogs and cats but exhibit low pathogenicity in cats (Clegg et al., 2012). In 1990, CPV-2a and CPV-2b were replaced by two new variants of CPV-2a (CPV-2a-297A) and CPV-2b (CPV-2b-297A), with one unique amino acid substitution, S297A (Decaro et al., 2009). In 2000, CPV-2c was first reported in Italy with one substitution at the VP2 gene (D426E) (Buonavoglia et al., 2001). Recently, CPV-2c has been circulating predominantly in Europe and the Americas (Decaro & Buonavoglia, 2012). CPV-2c has also been reported in Asia, including cases in China, India, Taiwan and Vietnam (Chiang, Wu, Chiou, Chang, & Lin, 2016; Nakamura et al., 2004; Nandi, Chidri, Kumar, & Chauhan, 2010; Zhao et al., 2016). It has also been reported that CPV-2c can cause severe diseases in cats (Miranda, Parrish, & Thompson, 2014; Nakamura et al., 2001). In Thailand, CPV-2a and CPV-2b have been reported as major variants circulating in dogs (Phromnoi, Sirinarumit, & Sirinarumit, 2010), while CPV-2c has never been reported in the country. In this study, CPV-2c was detected in domestic dogs and cats during a viral enteric disease surveillance. This study is the first to report and characterize an emergence of Asian-CPV-2c in domestic dogs and cats in Thailand.

## 2 | MATERIALS AND METHODS

From September 2016 to April 2018, the centre of excellence for emerging and re-emerging infectious diseases in animals (CUEIDAS), Chulalongkorn University, conducted a viral enteric disease surveillance of domestic dogs and cats in Thailand. The surveillance was carried out in four provinces of Thailand under the animal use and care protocol # 1731074. Rectal swab samples were mainly collected from dogs and cats with acute haemorrhagic or watery diarrhoea, vomiting, fever and dehydration. During 20 months of surveillance, 507 rectal swab samples were collected from dogs ( $n = 444$ ) and cats ( $n = 63$ ) of young age (<1 year), adult (1–5 years) and older (>5 years) with vaccination history records. Of 444 canine samples, 366 samples from sick dogs and 78 from healthy dogs were collected. Of 63 feline samples, 60 samples from sick cats and three from healthy animals were collected. All samples were subjected to parvovirus identification by PCR specific to the VP2 gene, as previously described (Buonavoglia et al., 2001).

For parvovirus identification, viral DNA was extracted from rectal swab samples by using the QIA Symphony DSP viral/Pathogen mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The viral DNA was stored at  $-20^{\circ}\text{C}$  until used. PCR assay for parvovirus identification was conducted as previously described (Buonavoglia et al.,

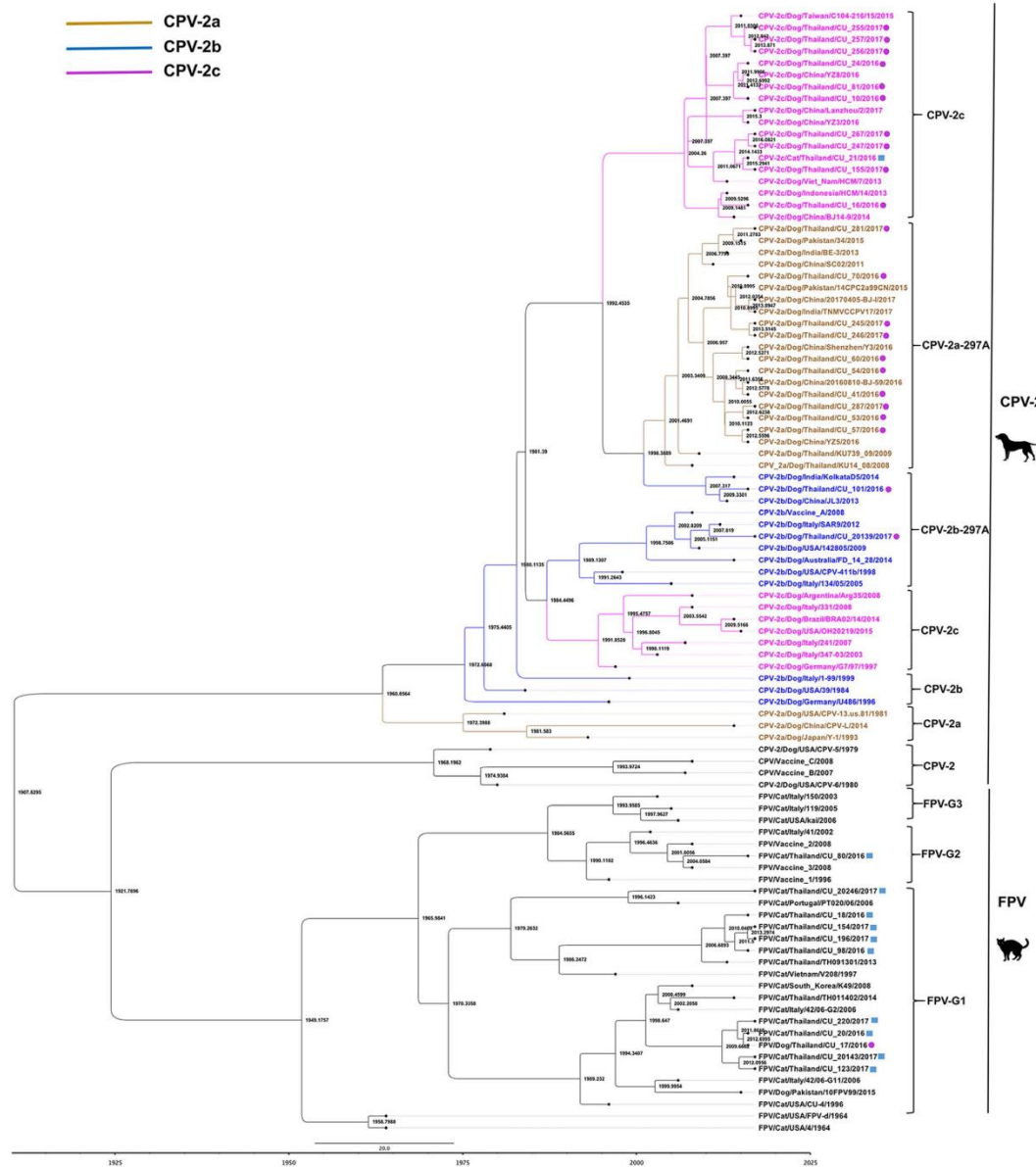
2001). The oligonucleotide primers specific to the VP2 gene were Hfor: 5'-CAGGTGATGAATTGCTACA-3' and Hrev: 5'-CATTGGATAAACTGGTG GT-3', located at positions 3556–3575 and 4166–4185 of CPV-2, respectively. In brief, PCR was performed in a final volume of 20  $\mu\text{l}$  comprising 1  $\mu\text{l}$  of DNA, 0.8  $\mu\text{M}$  of each forward and reverse primer, 1 $\times$  TopTaq Master Mix (Qiagen, Hilden, Germany), 1 $\times$  CoralLoad, and distilled water. The PCR condition was set as initial denaturation step at  $94^{\circ}\text{C}$  for 3 min 40 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $50^{\circ}\text{C}$  for 45 s and extension at  $72^{\circ}\text{C}$  for 1 min and final extension at  $72^{\circ}\text{C}$  for 7 min. The expected size of parvovirus positive amplified product was 611 bp. Identification of CPV2 antigenic variants was performed by using PCR-RFLP to differentiate CPV-2c and CPV-2a/CPV-2b variants. The PCR product size was 583 bp of the coding capsid protein VP2. Then, the PCR product was digested with enzyme Mbo II (New England Biolabs, USA) that selectively recognizes the restriction site "GAAGA" (nucleotide 4062–4066 of the VP2 encoding gene). The CPV-2c was digested into two fragments of 500 bp and 83 bp (Buonavoglia et al., 2001). The negative samples from CPV-2c PCR-RFLP assay were detected for CPV-2a and CPV-2b variants with specific primers (CPV-2abF/CPV-2abR and CPV-2bF/CPV-2bR) generating the product size of 681 bp and 427 bp, respectively (Pereira, Leal, & Durigon, 2007; Pereira, Monezi, Mehnert, D'Angelo, & Durigon, 2000) (Table S1). Concurrently, the CPV-2a/CPV-2b samples were confirmed by sequencing of the flanking region at amino acid position 426 to identify CPV-2a or CPV-2b variants.

For parvovirus characterization, we selected two parvoviruses (Dog/CU-24 and Cat/CU-21) for whole genome sequencing and the other 32 parvoviruses (CPV-2 = 21, FPV = 11) for VP2 gene sequencing. The criteria for selecting these 34 viruses for genetic characterization were based on epidemiological and demographic data, such as age of dog, date of isolation, breed and vaccination history. The selection criteria for the two viruses for whole genome sequencing were based on the representatives of CPV-2c from dogs (CU-24) and cats (CU-21). Parvovirus genome sequencing was conducted by using oligonucleotide primer sets previously described or new primer sets designed using the Primer 3 plus program (Table S1) (Buonavoglia et al., 2001; Koressaar & Remm, 2007; Untergasser et al., 2012). In brief, PCR was performed in a final volume of 30  $\mu\text{l}$  comprising 2  $\mu\text{l}$  of DNA, 0.4  $\mu\text{M}$  of each forward and reverse primer, 1 $\times$  TopTaq Master Mix, 1 $\times$  CoralLoad, and distilled water. The PCR condition was set as initial denaturation at  $94^{\circ}\text{C}$  for 3 min, 40 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $50^{\circ}\text{C}$  for 45 s, extension at  $72^{\circ}\text{C}$  for 2 min and final extension at  $72^{\circ}\text{C}$  for 7 min. PCR products were then purified and sequenced (1st Base Laboratories Sdn Bhd, Malaysia). Nucleotide sequences were assembled by using SeqMan software v.5.03 (DNASTAR Inc., Madison, WI).

For genetic analysis, pairwise comparison was conducted by using MegAlign software v.5.03 (DNASTAR Inc.). In brief, the nucleotide sequences and deduced amino acids of Thai-CPV-2

and FPV were aligned with those of vaccine and reference strains of CPV-2a, CPV-2b, CPV-2c, CPV-2a-297A, CPV-2b-297A from the USA (CPV-13/1981, CPV-411b/1998, OH20219/2015), Japan (Y1), China (SC-02/2011), India (KolkataD5/2014),

Indonesia (HCM14/2013), Italy (288-01/2001, 1-99/1999), Vietnam (HCM7/2013) and Thailand (KU14/2008). Genetic analysis for CPV-2 antigenic typing (VP2 at positions 297 and 426) and important amino acid determinants (VP2 at positions



**FIGURE 1** Phylogenetic tree of VP2 gene of canine parvovirus type 2 and feline parvovirus. Circles and squares represent Thai-CPV-2 and FPV, respectively. The phylogenetic tree was constructed by using the Beast program with Bayesian Markov-Chain Monte Carlo (BMCMC), with 10,000,000 generations and an average standard deviation of split frequencies <0.10. Values on branches represent times of most recent common ancestor (TMRCA) among CPV-2 antigenic types [Colour figure can be viewed at wileyonlinelibrary.com]



**TABLE 1** Association between age and clinical presentations of CPV-2 and FPV detection in this study

Age	Dogs		Cats	
	CPV-2 positive (%)		FPV positive (%)	
	Asymptomatic	Clinical sign	Asymptomatic	Clinical sign
Young (<1 year)	0/12 (0%)	91/198 (45.96%)	2/3 (66.67%)	28/47 (59.57%)
Adult (1–5 years)	3/63 (4.76%)	23/104 (22.12%)	0/0 (0%)	6/11 (54.55%)
Older (>5 years)	0/3 (0%)	16/64 (25.00%)	0/0 (0%)	1/2 (50.00%)
	3/78 (3.84%)	130/366 (35.52%)	2/3 (66.67%)	35/60 (58.33%)

**TABLE 2** Association between vaccine history and clinical presentations of CPV-2 and FPV detection in this study

Vaccine history	Dogs		Cats	
	CPV-2 positive (%)		FPV positive (%)	
	Asymptomatic	Clinical sign	Asymptomatic	Clinical sign
Non-vaccination	0/67 (0%)	103/231 (44.59%)	2/3 (66.67%)	34/53 (64.15%)
Completed	3/11 (27.27%)	27/135 (20.00%)	0/0 (0%)	1/7 (14.29%)
	3/78 (3.85%)	130/366 (35.52%)	2/3 (66.67%)	35/60 (58.33%)

300, 305, 321, 323, 324, 370, 371, 375) was conducted by the alignment of VP2 by using MEGA v6.06 and MegAlign software v.5.03 (DNASTAR Inc.). For the phylogenetic analysis, the partial VP2 gene sequences of Thai-CPV-2 and FPV were analysed with those of reference viruses. Vaccine and reference viruses, including CPV-2-vaccine strains ( $n = 3$ ), CPV-2 ( $n = 2$ ), CPV-2a ( $n = 3$ ), CPV-2b ( $n = 3$ ), CPV-2c ( $n = 14$ ), CPV-2a-297A ( $n = 11$ ), CPV-2b-297A ( $n = 7$ ), FPV vaccine ( $n = 3$ ), FPV-G1 ( $n = 9$ ), FPV-G2 ( $n = 1$ ) and FPV-G3 ( $n = 3$ ), were included in the phylogenetic analysis. The maximum clade credibility (MCC) tree of partial VP2 gene was constructed by BEAST 1.8 with the Bayesian Markov-Chain Monte Carlo (BMCMC) algorithm. A strict clock model with coalescent constant population and HKY with gamma 4 substitution were used as model parameters (Drummond, Suchard, Xie, & Rambaut, 2012). The Bayesian MCMC chain lengths were 10,000,000 generations, with sampling every 10,000 generations. The tree iteration was discharged with 10% of the chains as burn-in pattern by using a tree annotator, and the resulting MCC tree was drawn with FigTree software (v1.4.2) (Molecular evolution, phylogenetics and epidemiology, Edinburgh, Scotland, UK) (Figure 1). To determine the selective pressure on the partial VP2 (nucleotide positions 817–1314, amino acid positions 274–428), the ratio of non-synonymous (dN) to synonymous (dS) substitutions was estimated using Mixed Effects Model of Evolution (MEME) within the HyPhy software package (Murrell et al., 2012). The significance levels were set at  $p = 0.1$ . The values  $dN/dS > 1$ ,  $dN/dS = 1$  and  $dN/dS < 1$  were used to define positive selection, neutral mutations, and negative selection, respectively. A phylogenetic tree was also constructed by using maximum-likelihood with bootstrap analysis of 1,000 replications using the MEGA v.6.06 program (Tamura, Dudley, Nei, & Kumar, 2007) (Figure S1).

### 3 | RESULTS

From September 2016 to April 2018, a viral enteric disease surveillance of domestic dogs and cats was conducted in four provinces of Thailand. Of 444 canine samples and 63 feline samples subjected to parvovirus identification, the positivity of CPV-2 in dogs was 29.95% (133/444) and that of FPV in cats was 58.73% (37/63), which were high in non-vaccinated animals (44.59%). Moreover, animals of young age (<1 year) were more frequently infected with CPV-2 (45.96%) (Tables 1 and 2). In this study, all samples were also examined for other important enteric viruses, including canine rotavirus (CRV) and canine coronavirus (CoV). We found coinfection of CPV-2 and CRV ( $n = 1$ ) as well as CPV-2 and CoV ( $n = 22$ ) in dogs. Additionally, coinfection of FPV and CoV was observed in two cats (data not shown).

In this study, we identified antigenic types of CPV-2 as CPV-2c ( $n = 62$ ; 46.61%), CPV-2a ( $n = 68$ ; 51.13%) and CPV-2b ( $n = 3$ ; 2.26%) (Table S2). It is noted that both CPV-2c and CPV-2a were predominant variants and CPV-2c has never been reported in Thailand. In this study, we selected 34 parvoviruses for genetic characterization. For CPV-2, the viruses were subjected to VP2 gene ( $n = 21$ ) and whole genome sequencing ( $n = 2$ ; Dog/CU-24 and Cat/CU-21). For FPV, the viruses were subjected to VP2 gene sequencing ( $n = 11$ ). The nucleotide sequences of the parvoviruses were submitted to the GenBank database under accession no. MH711880–MH711913 (Table 3). Pairwise comparisons of nucleotide and deduced amino acid sequences of Thai viruses were performed against those of vaccine and reference strains. Our results showed that the whole genomes of two Thai-CPV-2 (Dog/CU-24 and Cat/CU-21) had 99.90% nucleotide identity to each other and the highest nucleotide identities to Vietnam CPV-2c (99.60% at WG, 99.90% at VP2)

**TABLE 3** Detailed descriptions of CPV-2 and FPV characterized in this study

Virus	Breed	Age of animal	Vaccine history	Clinical sign	Collection date	Location	Type of CPV/FPV	GenBank #
CPV								
Dog/Thailand/CU-41/2016	Mixed	2 years	C	Asymptomatic	Oct-16	Bangkok	CPV-2a-297A	MH711880
Dog/Thailand/CU-53/2016	Pomeranian	2 months	I	Diarrhoea	Oct-16	Bangkok	CPV-2a-297A	MH711881
Dog/Thailand/CU-54/2016	Yorkshire terrier	1 years	C	Diarrhoea	Oct-16	Bangkok	CPV-2a-297A	MH711882
Dog/Thailand/CU-57/2016	Pomeranian	2 months	I	Diarrhoea	Oct-16	Bangkok	CPV-2a-297A	MH711883
Dog/Thailand/CU-60/2016	Pomeranian	2 months	I	Diarrhoea	Oct-16	Bangkok	CPV-2a-297A	MH711884
Dog/Thailand/CU-70/2016	Siberian husky	4 months	I	Diarrhoea	Oct-16	Bangkok	CPV-2a-297A	MH711885
Dog/Thailand/CU-245/2017	Mixed	2 months	I	Diarrhoea	Apr-17	Bangkok	CPV-2a-297A	MH711886
Dog/Thailand/CU-246/2017	Beagle	2 months	I	Diarrhoea	Apr-17	Bangkok	CPV-2a-297A	MH711887
Dog/Thailand/CU-281/2017	Mixed	3 months	I	Diarrhoea	Sep-17	Bangkok	CPV-2a-297A	MH711888
Dog/Thailand/CU-287/2017	Mixed	1 year	I	Diarrhoea	Sep-17	Bangkok	CPV-2a-297A	MH711889
Dog/Thailand/CU-101/2016	Pekingese	2 months	I	Diarrhoea	Dec-16	Bangkok	CPV-2b-297A	MH711890
Dog/Thailand/ CU-20139/2017	Beagle	2 months	I	Diarrhoea	Nov-17	Bangkok	CPV-2b-297A	MH711891
Dog/Thailand/CU-10/2016	Beagle	2 years	C	Asymptomatic	Sep-16	Bangkok	CPV-2c	MH711892
Dog/Thailand/CU-16/2016	Shih Tzu	2 months	I	Diarrhoea	Sep-16	Bangkok	CPV-2c	MH711893
Dog/Thailand/CU-24/2016	Mixed	2 years	C	Asymptomatic	Oct-16	Bangkok	CPV-2c	MH711894 <sup>a</sup>
Dog/Thailand/CU-81/2016	Chihuahua	2 months	I	Diarrhoea	Nov-16	Bangkok	CPV-2c	MH711895
Dog/Thailand/CU-155/2017	Pomeranian	6 months	I	Diarrhoea	Jan-17	Bangkok	CPV-2c	MH711896
Dog/Thailand/CU-247/2017	Jack Russell	2 months	I	Diarrhoea	Apr-17	Bangkok	CPV-2c	MH711897
Dog/Thailand/CU-255/2017	German Shepherd	2 months	I	Diarrhoea	Jun-17	N.Ratchasima	CPV-2c	MH711898
Dog/Thailand/CU-256/2017	German Shepherd	2 months	I	Diarrhoea	Jun-17	N.Ratchasima	CPV-2c	MH711899
Dog/Thailand/CU-257/2017	German Shepherd	2 months	I	Diarrhoea	Jun-17	N.Ratchasima	CPV-2c	MH711900
Dog/Thailand/CU-267/2017	Mixed	4 months	I	Diarrhoea	Jul-17	Tak	CPV-2c	MH711901
Cat/Thailand/CU-21/2016	Mixed	5 months	I	Diarrhoea	Oct-16	Bangkok	CPV-2c	MH711902 <sup>a</sup>
FPV								
Cat/Thailand/CU-80/2016	Mixed	6 months	I	Diarrhoea	Nov-16	Bangkok	FPV-G2	MH711903
Cat/Thailand/CU-18/2016	Mixed	5 months	I	Diarrhoea	Sep-16	Bangkok	FPV-G1	MH711904
Cat/Thailand/CU-20/2016	Mixed	5 months	I	Diarrhoea	Sep-16	Bangkok	FPV-G1	MH711905
Cat/Thailand/CU-98/2016	Mixed	2 months	I	Diarrhoea	Dec-16	Bangkok	FPV-G1	MH711906
Cat/Thailand/CU-123/2017	Mixed	9 months	I	Asymptomatic	Jan-17	Chiang mai	FPV-G1	MH711907
Cat/Thailand/CU-154/2017	Mixed	3 months	I	Diarrhoea	Jan-17	Bangkok	FPV-G1	MH711908

(Continues)

TABLE 4 (Continued)

Virus	Breed	Age of animal	Vaccine history	Clinical sign	Collection date	Location	Type of CPV/FPV	GenBank #
Cat/Thailand/CU-196/2017	Mixed	1 year	I	Diarrhoea	Feb-17	Bangkok	FPV-G1	MH711909
Cat/Thailand/CU-220/2017	Mixed	3 months	I	Diarrhoea	Feb-17	Bangkok	FPV-G1	MH711910
Cat/Thailand/CU-20143/2017	Mixed	2 months	I	Diarrhoea	Nov-17	Bangkok	FPV-G1	MH711911
Cat/Thailand/CU-20246/2018	Mixed	5 months	I	Diarrhoea	Jan-18	Bangkok	FPV-G1	MH711912
Dog/Thailand/CU-17/2016	Labrador retriever	13 years	C	Diarrhoea	Sep-16	Bangkok	FPV-G1	MH711913

Note. <sup>a</sup>Whole genome sequence.

(Table S3). Within Thai-CPV-2, the VP2 gene is diverse with nucleotide identities of 99.80%–100% (CPV-2c), 99.00%–99.20% (CPV-2b-297A) and 98.80%–99.00% (CPV-2a-297A) (Table S4). In this study, the overall dN/dS ratio for the partial VP2 of CPV-2 and FPV was lower than 1 (0.296, 0.032), implying that the gene was under negative selection or purifying selection as the main evolutionary force.

Phylogenetic analysis of the VP2 gene from Thai-CPV-2 showed that the viruses were clustered with CPV-2c, CPV-2a-297A and CPV-2b-297A. The phylogenetic analysis indicated that Thai-CPV-2c was closely related to VietNam-HCM7, Chinese-YZ-8, BJ14-9, Taiwan-C104 and Indonesia-HCM but was in separate subclusters from the North American and European CPV-2c (Figure 1 and Figure S1). Based on the MCC tree, the Asian-CPV-2c was estimated to separate from CPV-2c of America and Europe since 1981. While, Thai-CPV-2c was started to evolved from other Asian-CPV-2c viruses (China, Taiwan, Vietnam and Indonesia) since 2004. The estimated nucleotide substitution rate of the partial VP2 was  $1.1905 \times 10^{-4}$  substitutions per site per year. 95% highest posterior densities (HPD) was  $6.9511 \times 10^{-5}$ – $1.6877 \times 10^{-4}$ . It is noted that the new variant CPV-2b-297A ( $n = 2$ ) was clustered in a separate group in which one isolate (Dog/CU-20139) was closely related to the vaccine strain (CPV-2b/Vaccine), suggesting a virus of vaccine origin. The phylogenetic analysis of the VP2 gene of FPV was also performed, showing that Thai-FPV was predominantly clustered with FPV-G1 ( $n = 10$ ), including one canine isolate (Dog/CU-17). In contrast, one Thai-FPV (Dog/CU-80) was grouped in a distinct cluster (G2) with FPV vaccine strains (Figure S1). It is interesting to note that one dog isolate was clustered with FPV-G1, suggesting FPV infection in a dog.

Genetic analyses of the genomes of Thai-CPV-2 and FPV were also conducted (Table 4). CPV-2a, CPV-2b and CPV-2c variants were determined by genetic differences at VP2 position 426 as Asn (N), Asp (D) and Glu (E), respectively (Martella, Decaro, & Buonavoglia, 2006). In this study, the new variants CPV-2a-297A and CPV-2b-297A, had unique amino acids at positions 297A, 426N and 426D, which were also observed in reference viruses. Similarly, Thai-CPV-2c contained unique amino acids at positions 297A and 426E, which were observed in reference CPV-2c. It is important to note that unique amino acid substitutions at positions Y324I and Q370R were only observed in the Asian strain CPV-2c (VietNam-HCM7, Chinese-YZ-8, BJ14-9, Taiwan-C104 and Indonesia-HCM), including Thai-CPV-2c, but were not observed in American and European CPV-2c (Table 4 and Figure 2).

#### 4 | DISCUSSION

To our knowledge, this study is the first to report CPV-2c in dogs and cats in Thailand. The infected animals showed clinical signs of acute haemorrhagic or watery diarrhoea. In this study, the positivity of CPV-2 in dogs was 29.95% and that of FPV in cats was 58.73%, which were high in non-vaccinated animals.



**TABLE 4** Genetic analysis of deduced amino acids of Thai-CPV-2 and FPV in comparison to those of vaccine and reference strains

Strain	Accession number	Year	Country	Amino acid position of VP2 gene												Type
				Typing	Important amino acids											
					297	426	300 <sup>a</sup>	305 <sup>a</sup>	321	323 <sup>b</sup>	324	370 <sup>c</sup>	371	375		
Reference CPV																
CPV-2/Dog/USA/CPV-5/1979	EU659116	1979	USA	S	N	A	D	N	N	N	N	Y	Q	A	N	CPV-2
CPV-2/Dog/USA/CPV-6/1980	EU659117	1980	USA	S	N	A	D	N	N	N	N	Y	Q	A	N	CPV-2
CPV-2/Vaccine B (Nobivac; Intervet)	FJ197846	2007	South Korea	S	N	A	D	N	N	N	N	Y	Q	A	N	CPV-2/Vaccine
CPV-2/Vaccine C (Vaccine06; Merial)	FJ222822	N/A	N/A	A	D	G	Y	K	N	N	N	Y	Q	A	D	CPV-2/Vaccine
CPV-2a/Dog/USA/CPV-13/1981	EU659118	1981	USA	S	N	G	Y	N	N	N	N	Y	Q	A	D	CPV-2a
CPV-2a/Dog/Japan/Y1/xxxx	D26079	N/A	Japan	S	N	G	Y	N	N	N	N	Y	Q	A	D	CPV-2a
CPV-2a/Dog/Thailand/KU14/2008	GQ379043	2008	Thailand	A	N	G	Y	N	N	N	N	I	Q	A	D	CPV-2a-297A
CPV-2a/Dog/China/SC02/2011	JX660690	2011	China	A	N	G	Y	N	N	N	N	I	Q	A	D	CPV-2a-297A
CPV-2b/Dog/Italy/1-99/1999	MF177226	1999	Italy	S	D	G	Y	N	N	N	N	Y	Q	A	D	CPV-2b
CPV-2b/Dog/USA/CPV-411b/1998	EU659121	1998	USA	A	D	G	Y	N	N	N	N	Y	Q	A	D	CPV-2b-297A
CPV-2b/Dog/India/KolkatabD5/2014	KP071953	2014	India	A	D	G	Y	N	N	N	N	I	Q	A	D	CPV-2b-297A
CPV-2b/Vaccine A (Duramune; Fort Dodge)	FJ222822	N/A	N/A	A	D	G	Y	K	N	N	N	Y	Q	A	D	CPV-2b/Vaccine
CPV-2c/Dog/Italy/288-01/2001	MF177239	2001	Italy	A	E	G	Y	N	N	N	N	Y	Q	A	D	CPV-2c
CPV-2c/Dog/USA/OH20219/2015	MF457594	2015	USA	A	E	G	Y	N	N	N	N	Y	Q	A	D	CPV-2c
CPV-2c/Dog/Vietnam/HCM/7/2013	LC214969	2013	Vietnam	A	E	G	Y	N	N	N	N	I	R	A	D	CPV-2c
CPV-2c/Dog/Indonesia/HCM/14/2013	LC216909	2013	Indonesia	A	E	G	Y	N	N	N	N	I	R	A	D	CPV-2c
CPV-2c/Dog/Taiwan/C104-216/2015	KX421787	2015	Taiwan	A	E	G	Y	N	N	N	N	I	R	A	D	CPV-2c
This study: CPV																
CPV-2a/Dog/Thailand/CU 41/2016	This study	2016	Thailand	A	N	G	Y	N	N	N	N	I	Q	A	D	CPV-2a-297A
CPV-2a/Dog/Thailand/CU 53/2016	This study	2016	Thailand	A	N	G	Y	N	N	N	N	I	Q	A	D	CPV-2a-297A
CPV-2a/Dog/Thailand/CU 54/2016	This study	2016	Thailand	A	N	G	Y	N	N	N	N	I	Q	A	D	CPV-2a-297A
CPV-2a/Dog/Thailand/CU 57/2016	This study	2016	Thailand	A	N	G	Y	N	N	N	N	I	Q	A	D	CPV-2a-297A
CPV-2a/Dog/Thailand/CU 60/2016	This study	2016	Thailand	A	N	G	Y	N	N	N	N	I	Q	A	D	CPV-2a-297A
CPV-2a/Dog/Thailand/CU 70/2016	This study	2016	Thailand	A	N	G	Y	N	N	N	N	I	Q	A	D	CPV-2a-297A
CPV-2a/Dog/Thailand/CU 245/2017	This study	2017	Thailand	A	N	G	Y	N	N	N	N	I	Q	A	D	CPV-2a-297A
CPV-2a/Dog/Thailand/CU 246/2017	This study	2017	Thailand	A	N	G	Y	N	N	N	N	I	Q	A	D	CPV-2a-297A
CPV-2a/Dog/Thailand/CU 281/2017	This study	2017	Thailand	A	N	G	Y	N	N	N	N	I	Q	A	D	CPV-2a-297A

(Continues)

TABLE 4 (Continued)

Strain	Accession number	Year	Country	Amino acid position of VP2 gene													Type
				Typing		Important amino acids											
				297	426	300 <sup>a</sup>	305 <sup>a</sup>	321	323 <sup>b</sup>	324	370 <sup>c</sup>	371	375				
CPV-2a/Dog/Thailand/CU 287/2017	This study	2017	Thailand	A	N	G	Y	N	N	N	I	Q	A	D	CPV-2a-297A		
CPV-2b/Dog/Thailand/CU 101/2016	This study	2016	Thailand	A	D	G	Y	N	N	N	I	Q	A	D	CPV-2b-297A		
CPV-2b/Dog/Thailand/CU 20139/2017	This study	2017	Thailand	A	D	G	Y	K	N	N	Y	Q	A	D	CPV-2b-297A		
CPV-2c/Dog/Thailand/CU 10/2016	This study	2016	Thailand	A	E	G	Y	N	N	N	I	R	A	D	CPV-2c		
CPV-2c/Dog/Thailand/CU 16/2016	This study	2016	Thailand	A	E	G	Y	N	N	N	I	R	A	D	CPV-2c		
CPV-2c/Dog/Thailand/CU 24/2016	This study	2016	Thailand	A	E	G	Y	N	N	N	I	R	A	D	CPV-2c		
CPV-2c/Dog/Thailand/CU 81/2016	This study	2016	Thailand	A	E	G	Y	N	N	N	I	R	A	D	CPV-2c		
CPV-2c/Dog/Thailand/CU 155/2017	This study	2017	Thailand	A	E	G	Y	N	N	N	I	R	A	D	CPV-2c		
CPV-2c/Dog/Thailand/CU 247/2017	This study	2017	Thailand	A	E	G	Y	N	N	N	I	R	A	D	CPV-2c		
CPV-2c/Dog/Thailand/CU 255/2017	This study	2017	Thailand	A	E	G	Y	N	N	N	I	R	A	D	CPV-2c		
CPV-2c/Dog/Thailand/CU 256/2017	This study	2017	Thailand	A	E	G	Y	N	N	N	I	R	A	D	CPV-2c		
CPV-2c/Dog/Thailand/CU 257/2017	This study	2017	Thailand	A	E	G	Y	N	N	N	I	R	A	D	CPV-2c		
CPV-2c/Dog/Thailand/CU 267/2017	This study	2017	Thailand	A	E	G	Y	N	N	N	I	R	A	D	CPV-2c		
CPV-2c/Cat/Thailand/CU 21/2016	This study	2016	Thailand	A	E	G	Y	N	N	N	I	R	A	D	CPV-2c <sup>d</sup>		
Reference FPV																	
FPV/Cat/USA-4/1964	EU659112	1964	USA	S	N	A	D	N	N	D	Y	Q	A	D			
FPV/Cat/USA/kail/2006	EU659115	2006	USA	S	N	A	D	N	N	D	Y	Q	A	D			
FPV/Cat/Italy/42/06-G2/2006	EU498698	2006	Italy	S	N	A	D	N	N	D	Y	Q	A	D			
FPV/Cat/Thailand/TH01402/2014	KT357494	2014	Thailand	S	N	A	D	N	N	D	Y	Q	A	D			
FPV/Dog/Pakistan/10FPV99/2015	MF182903	2015	Pakistan	S	N	A	D	N	N	D	Y	Q	A	D			
FPV/Vaccine 1 (PLI-IV)	D88287	N/A	N/A	S	N	A	D	N	N	D	Y	Q	A	D			
FPV/Vaccine 2 (Purevax;Merial)	EU498680	N/A	N/A	S	N	A	D	N	N	D	Y	Q	A	D			
FPV/Vaccine 3 (Felocell;Pfizer)	EU498681	N/A	N/A	S	N	A	D	N	N	D	Y	Q	A	D			
This study: FPV																	
FPV/Cat/Thailand/CU 80/2016	This study	2016	Thailand	S	N	A	D	N	N	D	Y	Q	A	D	FPV-G2		
FPV/Cat/Thailand/CU 18/2016	This study	2016	Thailand	S	N	A	D	N	N	D	Y	Q	A	D	FPV-G1		
FPV/Cat/Thailand/CU 20/2016	This study	2016	Thailand	S	N	A	D	N	N	D	Y	Q	A	D	FPV-G1		
FPV/Cat/Thailand/CU 98/2016	This study	2016	Thailand	S	N	A	D	N	N	D	Y	Q	A	D	FPV-G1		
FPV/Cat/Thailand/CU 123/2017	This study	2017	Thailand	S	N	A	D	N	N	D	Y	Q	A	D	FPV-G1		

(Continues)

TABLE 4 (Continued)

Strain	Accession number	Year	Country	Amino acid position of VP2 gene												
				Typing									Important amino acids			
				297	426	300 <sup>a</sup>	305 <sup>a</sup>	321	323 <sup>b</sup>	324	370 <sup>c</sup>	371	375	Type		
FPV/Cat/Thailand/CU 154/2017	This study	2017	Thailand	S	N	A	D	N	N	D	Y	Q	A	D	FPV-G1	
FPV/Cat/Thailand/CU 196/2017	This study	2017	Thailand	S	N	A	D	N	N	D	Y	Q	A	D	FPV-G1	
FPV/Cat/Thailand/CU 220/2017	This study	2017	Thailand	S	N	A	D	N	N	D	Y	Q	A	D	FPV-G1	
FPV/Cat/Thailand/CU 20143/2017	This study	2017	Thailand	S	N	A	D	N	N	D	Y	Q	A	D	FPV-G1	
FPV/Cat/Thailand/CU 20246/2017	This study	2017	Thailand	S	N	A	D	N	N	D	Y	Q	A	D	FPV-G1	
FPV/Dog/Thailand/CU 17/2016	This study	2016	Thailand	S	N	A	D	N	N	D	Y	Q	A	D	FPV-G1 <sup>b</sup>	

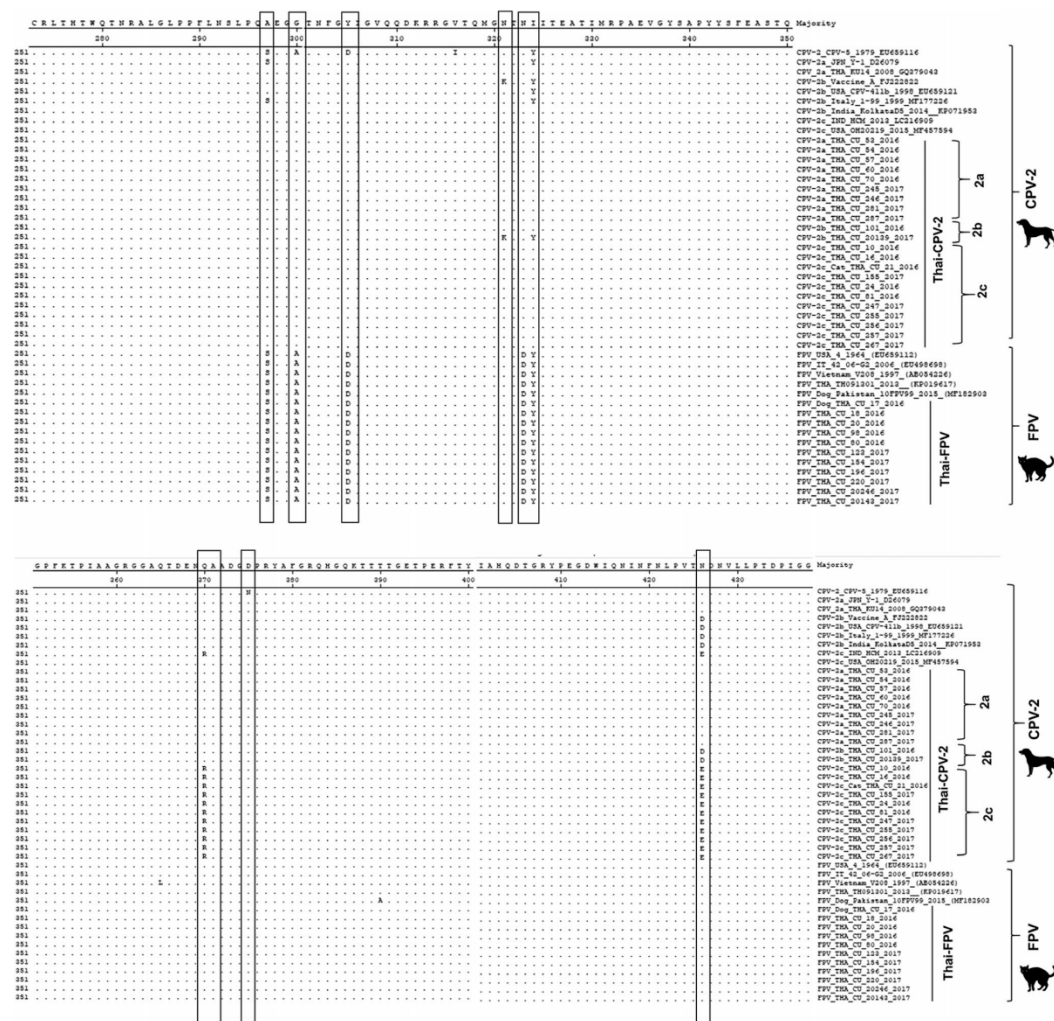
Notes: N/A not available.

<sup>a</sup>A300G, D305Y amino acid residue related to the adaptation of the CPV variants to the feline host (Ikeda et al., 2000; Truyen, Evermann, Vieler, & Parrish, 1996). <sup>b</sup>D323N amino acid residue related to specific canine host (binding with canine receptor (TfR) (Chang, Sgro, & Parrish, 1992; Govindasamy, Hueffer, Parrish, & Agbandje-Mckenna, 2003). <sup>c</sup>Q370R amino acid residue related to host range, novel Asian variant (Govindasamy et al., 2003).

This study also showed that CPV-2 was predominantly detected in dogs of young age (<1 year). These results were similar to the previous report of CPV-2 in puppies in Thailand (Sakulwira, Vanapongtipagorn, Theamboonlers, Oraveerakul, & Poovorawan, 2003). It is important to note that CPV-2c could also be isolated from cats. Similar observations were also reported in other studies (Miranda et al., 2014; Nakamura et al., 2001). One FPV infection in a dog was observed, as also seen in a previous study of FPV infection in sick dogs in Pakistan in 2018 (Ahmed et al., 2018).

Nucleotide and amino acid comparison showed that the whole genomes of two Thai-CPV-2 strains had 99.90% nucleotide identity to each other and had highest nucleotide identities to Asian-CPV-2c from Vietnam. Similar studies reported Asian-CPV-2c in China and Taiwan (Chiang et al., 2016; Guo et al., 2013). Phylogenetic analysis showed that Thai-CPV-2c is closely related to Asian-CPV-2c, including VietNam-HCM7, Chinese-YZ-8, BJ14-9, Taiwan-C104 and Indonesia-HCM. These viruses were in separate subclusters from North American and European CPV-2c. Our analysis suggested that the estimated time of the most recent common ancestor of Thai-CPV-2c subclusters was 2004 (Figure 1). The substitution rate of parvovirus in this study was in agreement with other studies ( $1.2 \times 10^{-4}$ – $2.2 \times 10^{-4}$  substitutions per site per year) (Hoelzer, Shackleton, Parrish, & Holmes, 2008; Pereira et al., 2007; Shackleton, Parrish, Truyen, & Holmes, 2005). Moreover, our data indicated that parvovirus (which is DNA virus) has high genomic substitution rate similar to other RNA viruses at approximately  $10^{-4}$  substitutions per site per year (Duffy, Shackleton, & Holmes, 2008). Whole genome analysis indicated that Thai-CPVs are closely related to Asian-CPV-2c with unique amino acids at position 297A, 370R and 426E of VP2, suggesting predominant Asian-CPV-2c in the country. It is also noted that unique amino acid substitutions at positions Y324I and Q370R were only observed in Asian strains of CPV-2c. These unique amino acids (370R) might relate to receptor-binding properties, suggesting species preference. Recent observations have also been reported in China and Taiwan (Chiang et al., 2016; Guo et al., 2013).

The identification of several types of CPV2 (CPV-2c, new variant CPV-2a-297A, and new variant CPV-2b-297A) demonstrates diversity of CPV2 in Thailand. CPV-2c is an emerging variant in the country and the Southeast Asia region. These findings will stimulate concern regarding whether currently used canine parvovirus vaccines will provide full protection against the new variant, Asian-CPV-2c. In summary, our results demonstrated the emergence of the new variant Asian-CPV-2c in dogs and cats in Thailand. Since cats can be infected with CPV-2c, dogs can also be infected with FPV. Thus, veterinary practitioners should focus more attention on both CPV and FPV infections, especially interspecies transmission. In Thailand, the surveillance of CPV and FPV should be further conducted on a larger scale to determine the dynamics of predominant variants and their distributions. This information will aid early diagnosis and the development of future strategies for domestic animal vaccination.



**FIGURE 2** Amino acid alignment of VP2 protein of CPV-2. Dots represent matched amino acid residues. Open boxes indicate amino acid substitutions

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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## APPENDIX E

**Molecular characterization identifies intra-host recombination and zoonotic potential of canine rotavirus among dogs from Thailand.**

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## ORIGINAL ARTICLE

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# Molecular characterization identifies intra-host recombination and zoonotic potential of canine rotavirus among dogs from Thailand

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**Abstract**

From September 2016 to January 2019, we collected 710 rectal swabs from both healthy and sick dogs from small animal hospitals in 5 provinces of Thailand. The samples were tested for canine rotavirus group A (CRV) by using one-step RT-PCR specific to the VP6 gene. Our results showed that 0.70% (5/710) were positive for CRV. The five CRVs were then characterized by whole-genome sequencing. Our results showed that the genotype of Thai CRVs is G3P[3], which is the predominant genotype reported in dogs. The Thai CRVs posed a novel genetic constellation 'G3-P[3]-I3-R3-C3-M3-A9-N2-T3-E3-H6', which has never been reported in CRVs from dogs but has been reported in rotaviruses from humans. Based on phylogenetic analysis, the Thai CRVs are the result of multiple reassortments in which gene segments might have originated from human and bat rotaviruses and suggests the zoonotic potential of the virus.

**KEYWORDS**

canine, characterization, rotavirus, Thailand, zoonotic

**1 | INTRODUCTION**

Rotavirus (RV) is an RNA virus belonging to the *Reoviridae* family. There are nine groups of rotaviruses (A-I). Group A rotavirus (RVA) is one of the major pathogens causing gastroenteritis in humans and animals worldwide (Greenberg & Estes, 2009). The virus contains 11 dsRNA segments encoding viral structure proteins (VP1, VP2,

VP3, VP4, VP6 and VP7) and non-structural proteins (NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6). The RVAs can be classified based on two classification systems. In the first classification system, two outer layer proteins (VP7 and VP4) are used to determine the genotype by G and [P]. There are 35G (G1-G35) and 50P (P[1]-P[50]) (Matthijnssens, Ciarlet, et al., 2011). Another classification system, the Rotavirus Classification Working Group (RCWG), classifies the

rotavirus genotype based on 11 genes as VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6, which presents as the acronym Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx to study evolution, the combination of interspecies transmission and reassortments between human and animal rotaviruses (Doro, Farkas, Martella, & Banyai, 2015).

Based on the complete genome analyses of RVAs, the viruses can be classified into three genotypes, genotype 1 (Wa-like), genotype 2 (DS-1-like) and genotype 3 (AU-1 like). The RVA genotypes 1 and 2 are the predominant genotypes circulating worldwide. Genotype 3 (AU-1 like) can be further classified into three genogroups; Cat 97-like genogroup (G3-P[3]-I3-R3-C2-M3-A9-N2-T3-E3-H6) (e.g. RO1845, HCR3A) (Tsugawa & Hoshino, 2008), AU-1-like genogroup (G3-P[9]-I3-R3-C3-M3-A3-N3-T3-E3-H3) (e.g. T152) (Rahman et al., 2007) and BA222-05-like genogroup (G3-P[9]-I2-R2-C2-M2-A3-N1/2-T6/3-E2-H3) (e.g. PAI58, PAH136) (Matthijnssens, De Grazia, et al., 2011).

In humans, RVAs were first detected in 1973 and have been reported worldwide. RVA causes around 500,000 deaths per year in children, especially in developing countries (Parashar et al., 2009). The common genotypes of RVAs in humans are G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8]. While G3P[3] and G3P[9] are rare genotypes and cause asymptomatic or mild gastroenteritis in humans (Ro1845, PA260/97 and CU-365). G3P[3] and G3P[9] in humans have been reported in several countries such as Brazil, Japan, India, Italy, Taiwan and Thailand (Banerjee et al., 2007; De Grazia, Giammanco, et al., 2007; De Grazia, Martella, et al., 2007; Degiuseppe, Silveyra, Reale, & Stupka, 2015; Khamrin, Peerakome, et al., 2006; Luchs, Cilli, Morillo, Carmona, & Timenetsky, 2012; Okitsu et al., 2018; Theamboonlers et al., 2013; Tsugawa & Hoshino, 2008).

In dogs, canine rotavirus (CRV) causes moderate-to-severe gastroenteritis disease (abdominal pain, mucoid diarrhoea and leucocytosis) in puppies younger than 2 weeks (Pollock & Carmichael, 1983). The prevalence of CRV is 2%–8% in dogs of a young age with gastroenteritis (Alves et al., 2018; Mochizuki, Hashimoto, & Ishida, 2001; Ortega, Martínez-Castañeda, Bautista-Gómez, Muñoz, & Hernández, 2017). A previous study reported a high prevalence (80%) of rotavirus antibodies in adult dogs (Rimmelzwaan et al., 1991). Rotavirus genotype G3P[3] is the predominant genotype in dogs in many countries, including Belgium, Japan, Hungary, Italy, South Korea and the United States (Kang et al., 2007; Matthijnssens, De Grazia, et al., 2011; Mihalov-Kovacs et al., 2015; Tsugawa & Hoshino, 2008).

Rotavirus is species specific, but cases of cross-species transmission from animals to humans via direct interspecies transmission or reassortment among viruses have been reported. Canine rotavirus might be less virulent in dogs, but it is likely to cause disease in humans including strains Ro1845 and PA260/97 (Tsugawa & Hoshino, 2008; Wu et al., 2012). It has been reported that the rotavirus genotypes G3P[9] (CU365) and G3P[3] (CMH222), which potentially originated from ruminants, cats and dogs, could be isolated from children with gastroenteritis in Thailand (Khamrin, Maneekarn, et al., 2006; Theamboonlers et al., 2013). In this study, we surveyed

CRVs in dogs in Thailand from September 2016 to January 2019. The Thai CRVs were characterized by whole-genome sequencing to determine the genotypes and possible multiple reassortment of the viruses.

## 2 | MATERIALS AND METHODS

### 2.1 | Sample collection

Sample collection from domestic dogs was conducted in small animal hospitals in 5 provinces of Thailand (Ayutthaya, Bangkok, Suphanburi, Nakhon Ratchasima and Tak) from September 2016 to January 2019. The 710 rectal swab samples were collected from healthy dogs ( $n = 93$ ) and dogs with gastroenteritis symptoms ( $n = 617$ ), including vomiting, watery diarrhoea, haemorrhagic diarrhoea and dehydration. The swab samples were collected from dogs of a young age ( $< 1$  year) ( $n = 389$ ) and dogs older than 1 year ( $> 1$  year) ( $n = 321$ ). The animals' demographic data, including age, sex, breed and vaccination history, were also recorded. The study was conducted under the Chulalongkorn University's Animal Use and Care Protocol # 1731074.

### 2.2 | Canine rotavirus detection

All 710 samples were subjected to canine rotavirus identification by one-step RT-PCR using primers specific to the VP6 gene of CRV. First, RNA extraction was performed using the QIA Symphony DSP Viral/Pathogen mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. To detect CRV, RNA samples were screened for CRV by using a one-step RT-PCR assay with the primers previously described, VP6F (5'-GACGGVGCRACTACATGGT-3') and VP6R (5'-GTCCAATTCATNCCTGGTGG-3') (Ortega et al., 2017). Briefly, one-step RT-PCR was conducted in a final volume of 50  $\mu$ l comprised of 3  $\mu$ l of template RNA, 25  $\mu$ l of 2xReaction Mix, 1.2  $\mu$ l of 10  $\mu$ M forward and reverse primers, 2.4  $\mu$ l of SuperScript III RT (Invitrogen, CA) and distilled water to a final volume of 50  $\mu$ l. 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 40 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 68°C for 1 min, as well as a final extension step at 68°C for 5 min. The expected size of the CRV-positive amplified products was 379 bp. Due to the dogs showing clinical signs similar to other canine viral enteric diseases, all samples were also tested for canine parvovirus, canine coronavirus and canine kobuvirus.

### 2.3 | Canine rotavirus characterization

In this study, five CRV-positive samples were subjected to whole-genome sequencing. Each viral gene was amplified using either



**TABLE 1** Description of the Thai CRVs characterized in this study

Viruses	Date	Location	Region	Age	Breed	Clinical signs	Accession number
RVA/Dog-wt/THA/CU126/2017/G3P[3]	Jan-2017	Tak	Northern	2 mts	Mixed	Asymptomatic	MT364824-34
RVA/Dog-wt/THA/CU128/2017/G3P[3]	Jan-2017	Tak	Northern	2 mts	Mixed	Asymptomatic	MT364835-45
RVA/Dog-wt/THA/CU132/2017/G3P[3]	Jan-2017	Tak	Northern	2 mts	Mixed	Asymptomatic	MT364846-56
RVA/Dog-wt/THA/CU20139/2017/G3P[3]	Nov-2017	Bangkok	Central	2 mts	Beagle	Diarrhoea	MT364857-67
RVA/Dog-wt/THA/CU23379/2019/G3P[3]	Jan-2019	Bangkok	Central	2 mts	German shepherd	Diarrhoea	MT364868-78

primers previously described or newly designed primer sets from the Primer 3 plus program (Koressaar & Remm, 2007; Tsugawa & Hoshino, 2008). Briefly, one-step RT-PCR was conducted in a final total volume of 50  $\mu$ l comprised of 3  $\mu$ l of template RNA, 25  $\mu$ l of 2xReaction Mix, 1.2  $\mu$ l of 10  $\mu$ M forward and reverse primers, 2.4  $\mu$ l of SuperScript III RT (Invitrogen, CA) and distilled water to a final volume of 50  $\mu$ l. 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, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 48–55°C for 30 s and extension at 68°C for 1–4 min, as well as a final extension step at 68°C for 5 min. PCR products were purified by NucleoSpin® Gel and PCR Clean-up (MACHEREY-NAGEL™, Germany). The purified PCR products were submitted to NovogeneAIT (Singapore) for HiSeq Illumina paired-end 50 cycles sequencing by using a NEBNext® UltraTM DNA kit for library preparation. The nucleotide sequences were assembled by trimmed-reads and by mapping all reads to the reference. Whole-genome sequences and the partial genome were extracted using a CLC genomic Workbench module 11.0 (Qiagen, Hilden, Germany).

Phylogenetic and genetic analyses were performed by comparing each gene of the CRV to the reference CRV and the other RV sequences available in the GenBank database. The reference nucleotide sequences included in the RV represent various geographical origins, host origins and date of isolations, and followed the previous studies (Grant et al., 2011; Okitsu et al., 2018; Sasaki et al., 2016; Theamboonlers et al., 2013). It should be noted that only seven complete genome sequences of CRV have been reported and included as reference CRVs in the databases (RV52-96/Italy, RV198-95/Italy, CU-1/USA, K9/USA, A79-10/USA, RS15/Japan and HUN135/Hungary). Then, the nucleotide sequences of each gene were aligned by using the Muscle program v.3.6. The evolution analysis was performed by using a BEAST 1.8 program applying a Bayesian Markov Chain Monte Carlo (BMCMC) algorithm. The best-fit substitution model was implemented by MEGA 7. A strict clock model with coalescent constant population and GTR, TN93 (G + I) and HKY with gamma 4 substitution were used as model parameters. The Bayesian MCMC chain lengths were 10,000,000–80,000,000 generations,

with sampling every 10,000 generations. The tree iteration was discharged with 10% of the chains as a burn-in pattern by using a tree annotator. The parameters were confirmed by calculating the effective sample size (ESS) with the TRACER program v. 1.7.1 (Molecular evolution, phylogenetics and epidemiology, Edinburgh, Scotland, UK). The minimum standard error in each gene segment was analysed by ESS values greater than 200. The resulting MCC tree was drawn with FigTree software (v1.4.2) (Molecular evolution, phylogenetics and epidemiology, Edinburgh, Scotland, UK).

### 3 | RESULTS

#### 3.1 | Canine rotavirus in dogs in Thailand

From September 2016 to January 2019, we collected 710 rectal swabs from both healthy and sick dogs from small animal hospitals in 5 provinces of Thailand. All rectal swab samples were tested for canine rotavirus group A by using one-step RT-PCR specific to the VP6 gene. Our results showed that 0.70% (5/710) were positive for CRV. RVA could be detected from both sick and asymptomatic dogs. The positive samples were all collected from young dogs of age <1 year. All five CRVs were then subjected to whole-genome sequencing and the nucleotide sequences of the Thai CRVs were submitted to the GenBank database under the accession number MT364824-78 (Table 1 and Table S1).

#### 3.2 | Genotype and the genetic constellation of Thai canine rotaviruses

In this study, five Thai CRVs designated RVA/Dog-wt/THA/CU126/2017/G3P[3] (CU126), RVA/Dog-wt/THA/CU128/2017/G3P[3] (CU128), RVA/Dog-wt/THA/CU132/2017/G3P[3] (CU132), RVA/Dog-wt/THA/CU20139/2017/G3P[3] (CU20139) and RVA/Dog-wt/THA/CU23379/2019/G3P[3] (CU23379) were characterized. The genotype of the CRVs was identified by the RotaC program (<http://rotac.regatools.be/>). The genetic

TABLE 2 The genetic constellation of the Thai CRVs and reference rotaviruses

Virus	Genotype <sup>a</sup>	Genogroup <sup>b</sup>	Gene										
			VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
This study													
RVA/Dog/THA/CU126/2017/ G3P[3]	AU-1	AU-1-like and Cat97-like	G3 <sup>c</sup>	P[3]	I3	R3	C3	M3	A9	N2	T3	E3	H6
RVA/Dog/THA/CU 128/2017/ G3P[3]	AU-1	AU-1-like and Cat97-like	G3	P[3]	I3	R3	C3	M3	A9	N2	T3	E3	H6
RVA/Dog/THA/CU 132/2017/ G3P[3]	AU-1	AU-1-like and Cat97-like	G3	P[3]	I3	R3	C3	M3	A9	N2	T3	E3	H6
RVA/Dog/THA/CU 20139/2017/ G3P[3]	AU-1	AU-1-like and Cat97-like	G3	P[3]	I3	R3	C3	M3	A9	N2	T3	E3	H6
RVA/Dog/THA/CU 23379/2019/ G3P[3]	AU-1	AU-1-like and Cat97-like	G3	P[3]	I3	R3	C3	M3	A9	N2	T3	E3	H6
Canine													
RVA/Dog-tc/ITA/RV52-96/1996/ G3P[3]	AU-1	Cat 97-like	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Dog-tc/ITA/RV198-95/1995/ G3P3	AU-1	Cat 97-like	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Dog-tc/USA/CU-1/1982/ G3P[3]	AU-1	Cat 97-like	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Dog-tc/USA/K9/1981/G3P[3]	AU-1	Cat 97-like	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Dog-tc/USA/A79-10/1979/ G3P[3]	AU-1	Cat 97-like	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Dog-tc/JPN/RS15/1982/ G3P[3]	AU-1	Cat 97-like	G3	P[3]	I3	R3	C2	M3	A9	N3	T3	E3	H6
Feline													
RVA/Cat-tc/AUS/Cat97/1984/ G3P[3]	AU-1	Cat 97-like	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Cat-wt/ITA/BA222/2005/ G3P[9]	AU-1	BA222-05-like	G3	P[9]	I2	R2	C2	M2	A3	N1	T3	E2	H3
Bat													
RVA/Bat-wt/ZMB/ LUS12-14/2012/G3P[3]	AU-1	Cat 97 and BA222-05-like	G3	P[3]	I3	R2	C2	M3	A9	N2	T3	E2	H3
Human													
RVA/Human-tc/JPN/AU-1/1982/ G3P[9]	AU-1	AU-1 like	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3

(Continues)

TABLE 2 (Continued)

Virus	Genotype <sup>a</sup>	Genogroup <sup>b</sup>	Gene										
			VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Human-tc/THA/T152/1998/ G12P[9]	AU-1	AU-1 like	G12	P[9]	I3	R3	C3	M3	A12	N3	T3	E3	H6
RVA/Human-tc/CHN/L621/2006/ G3P[9]	AU-1	AU-1 like	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H6
RVA/Human-wt/CHN/ E2451/2011/G3P[9]	AU-1	AU-1 like	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H6
RVA/Human-tc/THA/ CU-365/2008/G3P[9]	AU-1	AU-1 like	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H6
RVA/Human-wt/ITA/ PAH136/1996/G3P[9]	AU-1	BA222-05-like	G3	P[9]	I2	R2	C2	M2	A3	N1	T6	E2	H3
RVA/Human-wt/ITA/PA158/1996/ G3P[9]	AU-1	BA222-05-like	G3	P[9]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Human-tc/USA/ HCR3A/1984/G3P[3]	AU-1	Cat 97-like	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Human-tc/ISR/Ro1845/1985/ G3P[3]	AU-1	Cat 97-like	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Human-tc/ITA/ PA260-97/1997/G3P[3]	AU-1	AU-1-like and Cat97-like	G3	P[3]	I3	R3	C3	M3	A15	N2	T3	E3	H6
RVA/Human-wt/JPN/12638/2014/ G3P[3]	AU-1	AU-1-like and Cat97-like	G3	P[3]	I3	R3	C3	M3	A9	N2	T3	E3	H6
RVA/Human-wt/THA/ CMH222/2001/G3P[3]	AU-1	N/A	G3	P[3]	I8	-	-	-	-	-	-	E3	-
RVA/Human-tc/USA/Wa/1974/ G1P[8]	Wa	N/A	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-tc/USA/DS-1/1976/ G2P[4]	DS-1	N/A	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2

<sup>a</sup>RVA genotype based on a classification system of 11 gene segments can be classified into three genotypes; AU-1, Wa, and DS-1.

<sup>b</sup>AU-1 genotype can be classified into three genogroups; AU-1-like, Cat 97-like and BA222-05.

<sup>c</sup>Bold values present RVA genotype characterized in this study.

**TABLE 3** Nucleotide identities, genotype, nucleotide substitution rate and 95% posterior densities of each gene of the Thai CRVs

Gene	Genotype <sup>a</sup>	Closest RVs <sup>b</sup>	% nt identities <sup>b</sup> (% aa identities)	Nucleotide substitution rate/site/year	95% posterior densities (HPD)	Potential origin at tMRCA <sup>c</sup>	tMRCA <sup>c</sup>
VP7	G3	Human RVA (12638/Japan)	98.80% (99.30%)	$5.1127 \times 10^{-4}$	$3.343 \times 10^{-4}$ – $6.9163 \times 10^{-4}$	Human RVA (R2638/Brazil)	1989
VP4	P[3]	Bat RVA (LUS1214/Zambia)	97.30% (98.00%)	$4.5008 \times 10^{-4}$	$3.4495 \times 10^{-4}$ – $5.537 \times 10^{-4}$	Bat RVA (LUS1214/Zambia)	1991
VP1	R3	Human RVA (12638/Japan)	97.40% (99.40%)	$4.63517 \times 10^{-4}$	$3.5527 \times 10^{-4}$ – $5.174 \times 10^{-4}$	Human RVA (12638/Japan)	1994
VP2	C3	Human RVA (12638/Japan)	98.80% (100%)	$2.3015 \times 10^{-4}$	$1.4649 \times 10^{-4}$ – $3.0954 \times 10^{-4}$	Human RVA (12638/Japan; L621/China)	1995
VP3	M3	Human RVA <sup>d</sup> (12638/Japan) Bat RVA <sup>e</sup> (LUS1214/Zambia)	98.50% (98.60%) 98.80% (99.20%)	$3.2645 \times 10^{-4}$	$2.225 \times 10^{-4}$ – $4.2199 \times 10^{-4}$	Human RVA (12638/Japan) Bat RVA (LUS1214/Zambia)	1997 1966
VP6	I3	Human RVA (12638/Japan)	99.20% (99.50%)	$3.6436 \times 10^{-4}$	$2.186 \times 10^{-4}$ – $5.1202 \times 10^{-4}$	Human RVA (12638/Japan)	2000
NSP1	A9	Bat RVA (LUS1214/Zambia)	98.10% (98.60%)	$3.0709 \times 10^{-4}$	$1.5829 \times 10^{-4}$ – $4.6072 \times 10^{-4}$	Bat RVA (LUS1214/Zambia)	1985
NSP2	N2	Bat RVA (LUS1214/Zambia)	98.30% (98.40%)	$5.3834 \times 10^{-4}$	$3.766 \times 10^{-4}$ – $7.0075 \times 10^{-4}$	Bat RVA (LUS1214/Zambia) Human RVA (12638/Japan)	1995
NSP3	T3	Cat RVA <sup>f</sup> (FRV348/Japan) Human RVA <sup>g</sup> (12638/Japan)	96.70% (97.50%) 99.00% (99.40%)	$4.5655 \times 10^{-4}$	$3.0257 \times 10^{-4}$ – $6.1026 \times 10^{-4}$	Cat RVA (FRV348/Japan) Human RVA (12638/Japan)	1965 2002
NSP4	E3	Human RVA (12638/Japan)	97.70% (98.80%)	$5.2603 \times 10^{-4}$	$2.6643 \times 10^{-4}$ – $8.0098 \times 10^{-4}$	Human RVA (12638/Japan)	1992
NSP5	H6	Human RVA (12638/Japan)	97.70% (98.00%)	$4.1703 \times 10^{-4}$	$2.7526 \times 10^{-5}$ – $6.455 \times 10^{-4}$	Human RVA (12638/Japan)	1988

<sup>a</sup>RVA genotype based on classification of outer layer protein genes (VP7 and VP4).

<sup>b</sup>nt identities and aa identities based on BLAST analysis.

<sup>c</sup>Results are based on MCC phylogenetic analysis.

<sup>d</sup>VP3 gene of Thai CRVs (CU20139) posed highest nucleotide identities with human RVA (12638/Japan).

<sup>e</sup>VP3 gene of Thai CRVs (CU126, CU128, CU132 and CU 23379) posed highest nucleotide identities with bat (LUS12-14/Zambia).

<sup>f</sup>NSP3 gene of Thai CRVs (CU20139) posed highest nucleotide identities with cat RVA (FRV348/JPN).

<sup>g</sup>VP3 gene of Thai CRVs (CU126, CU128, CU132 and CU 23379) posed highest nucleotide identities with human RVA (12638/Japan).

constellation of five Thai CRVs was G3-P[3]-I3-R3-C3- M3-A9-N2-T3-E3-H6. Then, we compared the genetic constellation of the Thai CRVs to the reference RVs from cats, dogs, bats, pigs, horses and humans. Our results showed that the Thai CRVs were identical to Human RVA in 2014 (RVA/Human-wt/JPN/12638/2014/G3P[3]), which has the motif G3-P[3]-I3-R3-C3- M3-A9-N2-T3-E3-H6 (nucleotide identities ranging from 92.90%–99.20%). It should be noted that the Thai CRVs belong to genotype AU-1 with gene segments of both genogroup Au-1-like and Cat 97-like, which have never been reported before for any canine rotaviruses (Table 2 and Table S2).

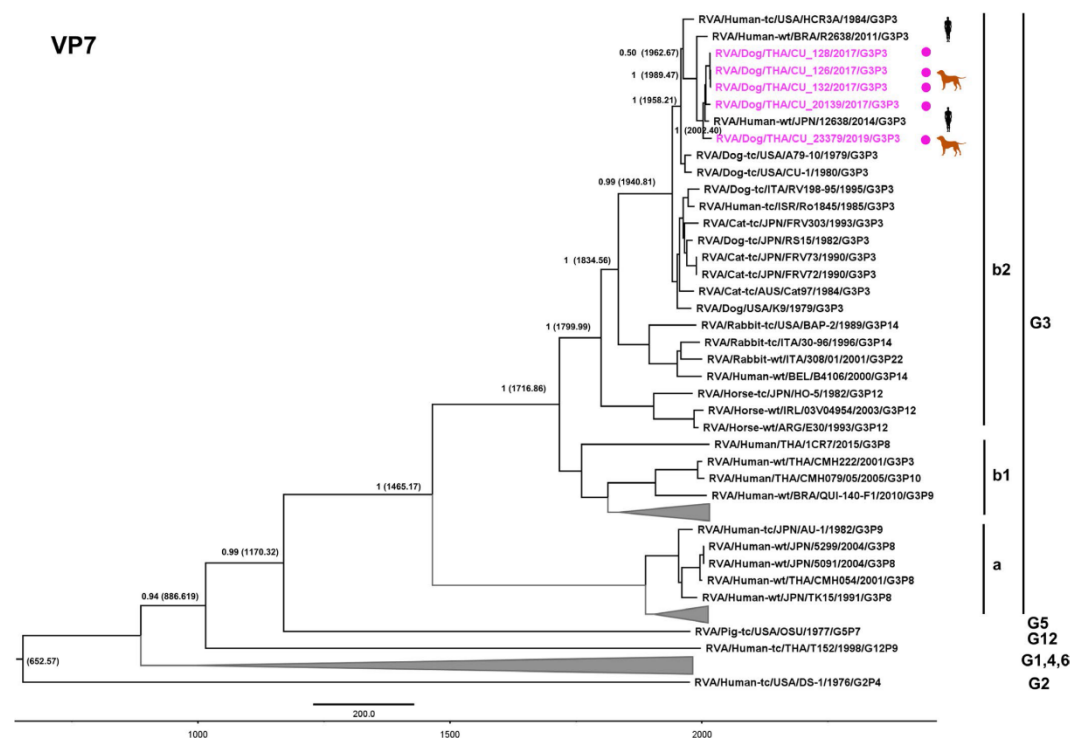
### 3.3 | Genotype G3P[3] of the Thai Canine Rotaviruses

Based on nucleotide identities and phylogenetic analysis of the VP7 and VP4 genes, our results showed that the Thai CRVs belonged to the genotype G3P[3]. For the VP7 gene, the Thai CRVs ( $n = 5$ ) possessed high nucleotide identities to human RVA (12638/Japan) at 98.80% (99.30% aa identities). The nucleotide identities of the VP7 gene of the Thai CRVs compared to that of the other RVAs of the G3 genotype from horses, bats, pigs, cats and dogs ranged from 79.50% to 95.70% (92.10%–98.60% aa identities).

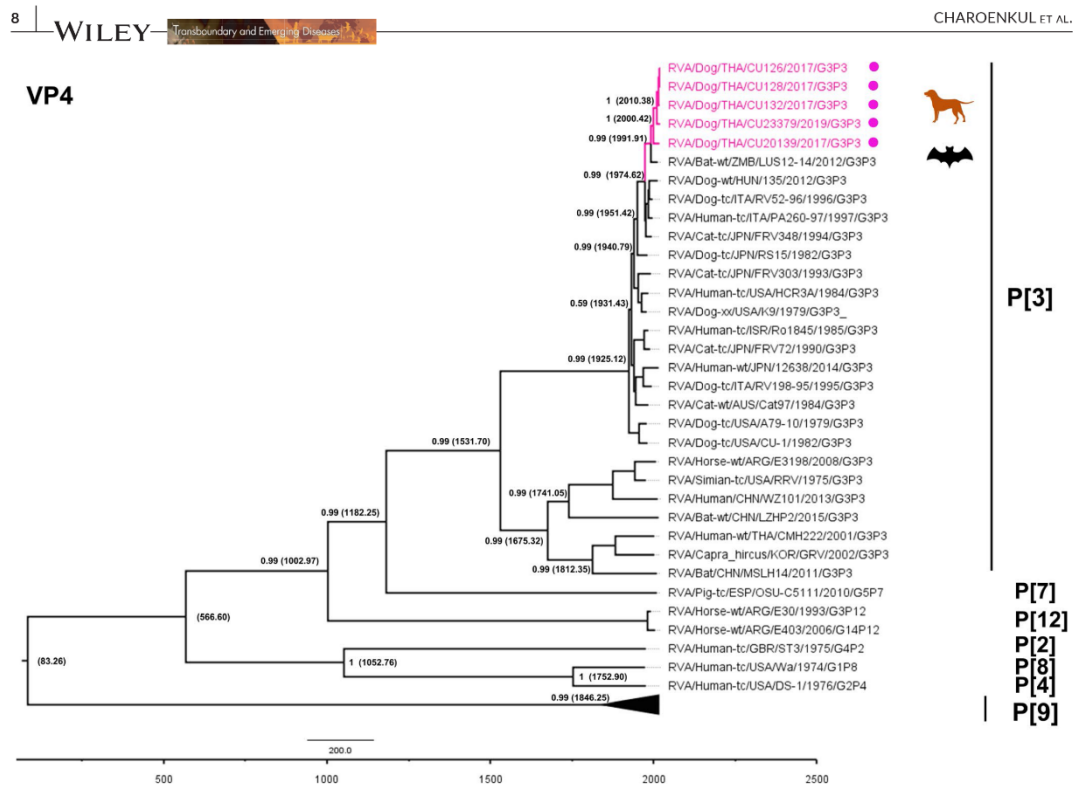
The phylogenetic tree of the VP7 of the RVAs showed that genotype G3 could be divided into 2 major clusters, A and B, and subclusters b1 and b2. The Thai CRVs were grouped with subcluster b2 and were closely related to human RVA (12638/Japan, R2638/Brazil and HCR3A/USA). Based on the MCC phylogenetic tree, the Thai CRVs were estimated to have separated from human rotavirus (R2638/Brazil) since 1989 (Table 3 and Figure 1). The estimated nucleotide substitution rate of VP7 was  $5.1127 \times 10^{-4}$  substitutions per site per year (95% posterior densities (HPD);  $3.343 \times 10^{-4} - 6.9163 \times 10^{-4}$ ). For the VP4 gene, the Thai CRVs had highest nucleotide identities to bat RVA (LUS12-14/Zambia) at 97.30% nt identities (98.00% aa identities). Comparing to the other P[3] RVs of horses, bats, dogs, cats and humans, the Thai CRVs had 80.70%-96.90% nt identities (88.30% -97.60% aa identities). The phylogenetic tree of VP4 showed that the Thai CRVs were grouped into the P[3] cluster. MCC tree analysis showed that the Thai CRVs likely diverged from bat RVA (LUS12-14/Zambia) since 1991. The estimated nucleotide substitution rate of VP4 was  $4.5008 \times 10^{-4}$  substitutions per site per year (95% posterior densities (HPD);  $3.4495 \times 10^{-4} - 5.537 \times 10^{-4}$ ) (Table 3 and Figure 2).

Phylogenetic analysis of the other structural proteins (VP) and non-structural proteins (NSP) are shown in Table 3 and the supplement Figures. For VP1, VP2 and VP6, the Thai CRVs possessed the highest nucleotide identities to human rotavirus (12638/JPN). The phylogenetic trees showed that the Thai CRVs were grouped into the R3, C3 and I3 groups. The tMRCA showed that the VP1, VP2 and VP6 of the Thai CRVs were estimated to have separated from human rotavirus (12638/Japan) in the 1990s. The VP3 gene of the Thai CRVs (CU20139) had the highest nucleotide identities with human RVA (12638/Japan). The Thai CRVs (CU126, CU128, CU132 and CU 23379) had the highest nucleotide identities with bat RVA (LUS12-14/Zambia). The tMRCA revealed that the Thai CRVs (CU126, CU128, CU132 and CU 23379) had diverged from bat RVA (LUS12-14/Zambia) in 1966 and the Thai CRV (CU20139) from human RVA (12638/Japan) in 1997 (Table 3, Figure 3 and Figures S1-S3).

For NSP1 and NSP2, the Thai CRVs had the highest nucleotide identities with a bat RVA (LUS12-14/Zambia). The MCC tree showed that the Thai CRVs were grouped into A9 and N2 groups and originated from bat RVA (LUS12-14/Zambia) and human RVA (12638/



**FIGURE 1** Phylogenetic tree of VP7. The maximum clade credibility (MCC) tree was constructed by BEAST 1.8 with the Bayesian Markov Chain Monte Carlo (BMCMC) algorithm. A strict clock model with a coalescent constant population and GTR, TN93 (G + I) and HKY with a gamma 4 substitution were used as model parameters. Pink circles indicate the Thai CRVs characterized in this study. Dog images (orange) represent the Thai CRVs and human images (black) represent reference human RVs closely related to the Thai CRVs



**FIGURE 2** Phylogenetic tree of VP4. The maximum clade credibility (MCC) tree was constructed by BEAST 1.8 with the Bayesian Markov Chain Monte Carlo (BMCMC) algorithm. A strict clock model with a coalescent constant population and GTR, TN93 (G + I) and HKY with a gamma 4 substitution were used as model parameters. Pink circles indicate the Thai CRVs characterized in this study. Dog images (orange) represent the Thai CRVs and bat images (black) represent the reference bat RVs closely related to the Thai CRVs

Japan) during 1985–1995. For NSP3, the Thai CRV (CU20139) had the highest nucleotide identities with cat RVA (FRV348/JPN). The other Thai CRVs (CU126, CU128, CU132 and CU23379) had the highest nucleotide identities with human RVA (12638/Japan). The MCC tree of NSP3 showed that the Thai CRV (CU20139) was diverged from the cat RVA (strain FRV348/Japan) since 1965. The other Thai CRVs (CU126, CU128, CU132 and CU23379) were likely diverged from human RVA (12638/Japan) in 2002. For NSP4 and NSP5, the Thai CRVs had the highest nucleotide identities with human RVA (12638/Japan). The MCC tree of NSP4 and NSP5 showed that the Thai CRVs were grouped into the E3 and H6 groups and likely diverged from human RVA (12638/Japan) since 1988–1992 (Table 3 and Figures S4–S8).

### 3.4 | Genetic analysis of Thai canine rotaviruses

Genetic analyses of the nucleotide and deduced amino acids of the Thai CRVs were conducted by comparing the Thai CRVs against reference RVAs from humans, dogs, cats, bats, and vaccine strains.

The antigenic epitopes of the G3 genotype at regions 7-1a, 7-1b and 7-2, correlating with the blockade of neutralizing antibodies, were analysed. The Thai CRVs contained amino acid substitutions in region 7-1b (A212V, N213S, K238D and D242T) and region 7-2 (T147A and A221T) which are also observed in reference rotaviruses from dogs and humans (HCR3A/USA, Ro1845/Israel, R2638/Brazil and 12638/Japan), but are different from the vaccine strain (Wi78-9 (RotaTeq™)) (Table 4). The Thai CRVs also had unique amino acids at positions 18F, 22M, 212V, and 221T which are only observed in reference viruses of the genotype G3, subcluster b2. The unique amino acids at positions 16F, 49K, 68A, 121E and 238D were also found in the RVAs of cluster B (both subcluster b1 and b2), which can be used to differentiate cluster A and cluster B (Table S3).

## 4 | DISCUSSION

To our knowledge, this study is the first to report on the genetics of canine rotavirus (CRV) in dogs in Thailand. Up to date, there are only seven complete genomes of canine rotavirus group A of genotype



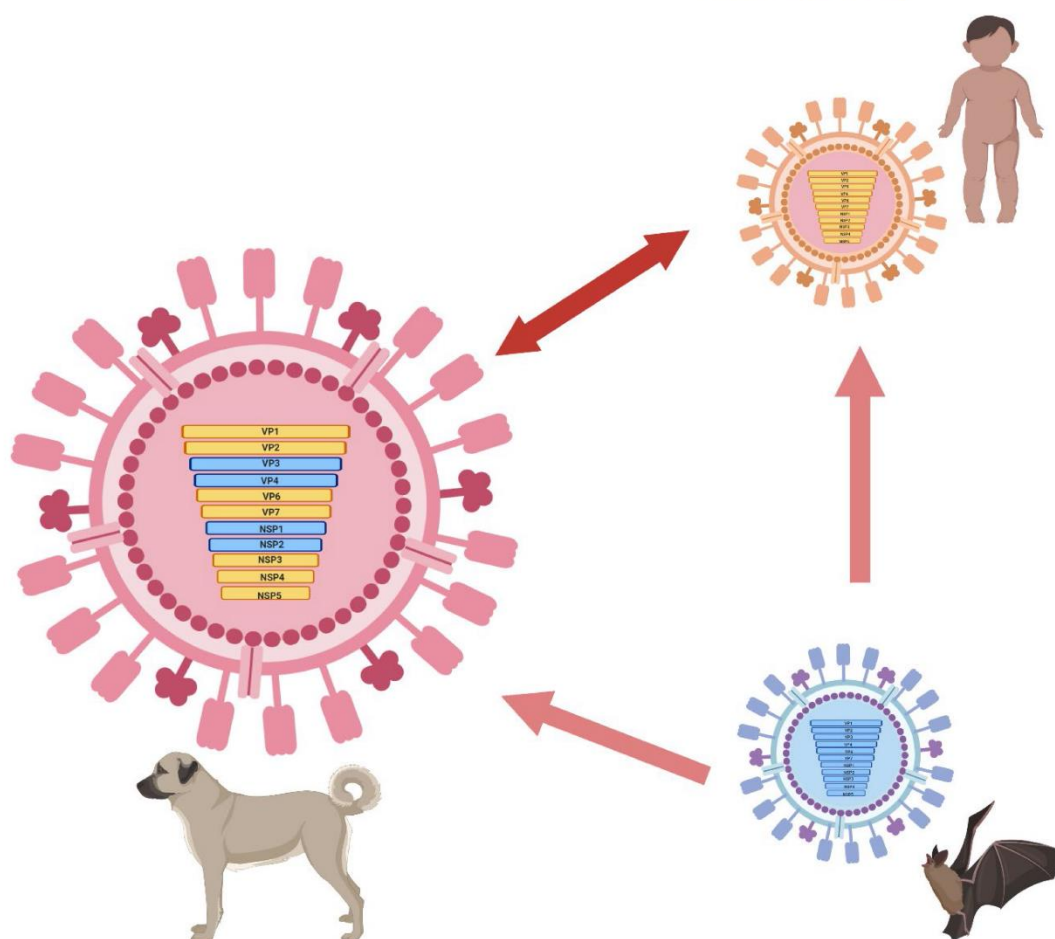


**TABLE 4** Genetic analysis of antigenic regions at VP7 among the Thai CRVs and reference RVAs from dogs, cats, bats, humans and vaccine strains

Viruses	Amino acid position																																				
	7-1a region							7-1b region							7-2 region																						
	87	91	94	96	97	98	99	100	104	123	125	129	130	201	211	212	213	238	242	143	145	146	147	148	190	217	221	264									
Vaccine strain																																					
WI78-9 (Rotateq™) (G3) <sup>b</sup>	USA	1992	T	T	N	N	S	W	K	D	Q	D	A	V	D	Q	D	A	N	K	D	A	N	K	D	K	D	A	T	L	S	E	A	G			
This study																																					
CU 20139	THA	2017	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
CU 126	THA	2017	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
CU 128	THA	2017	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
CU 132	THA	2017	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
CU23379	THA	2019	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Canine																																					
K9	USA	1979	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
A79-10	USA	1979	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Feline																																					
Cat97	AUS	1984	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BA222	ITA	2005	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bat																																					
LUS12-14	ZMB	2012	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Human																																					
AU-1	JPN	1982	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HCR3A	USA	1984	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ro1845	ISSR	1985	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
1A3739	BRA	2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R2638	BRA	2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
12638	JPN	2014	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMH222	THA	2001	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CU365-KK	THA	2008	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

<sup>a</sup>Identical amino acid with vaccine strain (Rotarix-A41CA419A) is represented by dots.  
<sup>b</sup>Zeller et al. (2012).





**FIGURE 4** Schematic presentation of possible multiple reassortment of the Thai CRVs among the dog, bat and human rotaviruses

reassortment of genes among humans and animals RVAs (Figure 4). However, a limitation of this study is the limited data of reference CRVs available in the GenBank database and only five positive samples were characterized in this study. Additionally, there are no information about contact history among human, bat and dog. Thus, this study does not provide conclusive evidence of cross-species transmission of CRV. Since our analysis was based on the available sequence data from the GenBank, if expansions of the nucleotide sequences of RVAs from various animal species become available, it would help us to better understand the interspecies transmission and multiple reassortment of these viruses in the future.

In this study, the nucleotide substitution rates of all segments of the Thai CRVs are  $2.3015\text{--}5.3834 \times 10^{-4}$  (95%HPD:  $1.4649\text{--}8.0098 \times 10^{-4}$ ) (Table 4), which is similar to the estimated rate from other studies ( $9.7 \times 10^{-4}\text{--}4.1 \times 10^{-3}$ ) (Fujii et al., 2019; Jere

et al., 2018). Moreover, our data indicated that the Thai CRVs had a higher genomic substitution rate than other dsRNA, approximately  $1 \times 10^{-5}$  substitutions per site per year (Firth et al., 2010). This result suggests the rapid evolution of the virus, and interspecies transmission of rotavirus is one of the factors activating this evolutionary mechanism. For example, the RVA genotype G3P[3] in humans has been reported worldwide such as in Brazil (R2638), Italy (PA260-97), United States (HCR3A), Israel (Ro1845), Japan (12638) and Thailand (CMH222), and the genetic constellation of G3P[3] contains genes with an animal origin (De Grazia, Martella, et al., 2007; Khamrin, Maneekarn, et al., 2006; Luchs et al., 2012; Okitsu et al., 2018; Tsugawa & Hoshino, 2008).

The genetic analysis result showed that the Thai CRVs posed characteristics of lineage b2 RVA. The analysis of antigenic epitopes of VP7 showed that the Thai CRVs posed a unique amino

acid at region 7-1b (A212V, N213S, K238D and D242T) and region 7-2 (T147A and A221T) similar to canine and human RVAs (HUN/135, HCR3A/USA and 12638/Japan) but different from the commercial human rotavirus vaccine (Wi78-9 (RotaTeq™)). These unique amino acids are related to neutralizing antibodies and rotavirus vaccine efficacy (Dennehy, 2008; Zeller et al., 2012). The unique amino acids at these positions (16F, 49K, 68A, 121E, 238D) could be found in both subclusters b1 and b2, which can be used to differentiate clusters A and B. This observation has also been reported in a previous study in Japan (Okitsu et al., 2018). The Thai CRVs contained unique amino acids at positions 18F, 22M, 212V and 221T, suggesting unique amino acid determinants of lineage b2, which can be further used for diagnostic and genotyping purposes. However, the important and unique amino acids need further investigation.

In summary, this study is the first to report canine rotaviruses genotype G3P[3] in dogs in Thailand. The Thai CRVs belonged to genotype AU-1 with gene segments of both genogroup Au-1-like and Cat 97-like, which have never been reported before in any canine rotaviruses. Phylogenetic analyses showed that the Thai CRVs were closely related and might have originated from human and bat RVAs with multiple reassortment. Thus, dog owners and veterinary practitioners should pay more attention to rotavirus infection as a potentially zoonotic and reverse zoonotic viral disease. There is a need to survey CRVs on a larger scale to determine the dynamics and distribution of rotaviruses in Thailand.

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#### CONFLICT OF INTEREST

All authors declare no conflicts of interest.

#### ETHICS APPROVAL

This study was conducted under the approval of the Institute for Animal Care and Use Protocol of the CU-VET, Chulalongkorn University (IACUC # 1731074).

#### DATA AVAILABILITY STATEMENT

The nucleotide sequence data that support the findings of this study are openly available in the GenBank database at <https://www.ncbi.nlm.nih.gov/genbank/>, RVA/Dog-wt/THA/CU126/2017/G3P[3] (MT364824-MT364834), RVA/Dog-wt/THA/CU128/2017/G3P[3]

(MT364835-MT364845), RVA/Dog-wt/THA/CU132/2017/G3P[3] (MT364846-MT364856), RVA/Dog-wt/THA/CU20139/2017/G3P[3] (MT364857-MT364867), and RVA/Dog-wt/THA/CU23379/2019/G3P[3] (MT364868-MT364878).

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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