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DEVELOPMENT OF MULTIPLEX RT-PCR AND METAGENOMIC ANALYSES OF CANINE INFECTIOUS RESPIRATORY DISEASE COMPLEX ASSOCIATED VIRUSES IN DOGS

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ฉัตรชัย ผิวบาง : การพัฒนาปฏิกิริยาลูกโซ่โพลีเมอเรสแบบมัลติเพล็กซ์ และการวิเคราะห์สารพันธุกรรมด้วยกระบวนการเมตาจีโนมิกซ์ในโรคระบบทางเดินหายใจสุนัขแบบซับซ้อนที่เกิดเนื่องจากไวรัส (DEVELOPMENT OF MULTIPLEX RT-PCR AND METAGENOMIC ANALYSES OF CANINE INFECTIOUS RESPIRATORY DISEASE COMPLEX ASSOCIATED VIRUSES IN DOGS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. สพ.ญ. ดร. สมพร เตชะงามสุวรรณ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. น.สพ. ดร. อนุเทพ รังสีพิพัฒน์, ศ. นพ. ยง ภู่วรวรรณ, 185 หน้า.

กลุ่มอาการโรคติดเชื้อในระบบทางเดินหายใจสุนัขแบบซับซ้อน เป็นโรคที่มีความสัมพันธ์กับหลายสาเหตุ อันได้แก่ความไวรับของสัตว์ ปัจจัยทางสภาวะแวดล้อม และความรุนแรงของเชื้อในการก่อโรค เชื้อไวรัสที่มีการก่อโรคระบบทางเดินหายใจแบบซับซ้อนในสุนัขได้แก่ เชื้อไข้หวัดสุนัขชนิดอินฟลูเอ็นซ่า (canine influenza virus; CIV) เชื้อไข้หวัดสุนัขชนิดพาราอินฟลูเอ็นซ่า (canine parainfluenza virus; CPIV) เชื้อไข้หัดสุนัข (canine distemper virus; CDV) เชื้อโคโรน่าระบบทางเดินหายใจ (canine respiratory coronavirus; CRCoV) เชื้ออะดีโนไวรัส ชนิดที่ ๒ (canine adenovirus type 2; CAdV-2) และ เชื้อไวรัสเฮอร์ปี้ซ์ชนิดที่ ๑ (canine herpesvirus 1; CaHV-1) นอกจากชนิดของเชื้อก่อโรคแล้ว ปัจจัยทางสิ่งแวดล้อม ถือเป็นปัจจัยสำคัญต่อการเกิดโรค โดยเฉพาะโอกาสในการติดเชื้อ ซึ่งโดยส่วนใหญ่มักติดเชื้อผ่านทางการหายใจ โดยช่องทางในการติดต่อสามารถแบ่งแยกตามสภาพแวดล้อมออกเป็น การติดเชื้อจากชุมชน (community-acquired infection; CAI) และการติดเชื้อจากโรงพยาบาลสัตว์ (hospital-associated infection; HAI) ซึ่งในทางการสัตวแพทย์ ยังไม่มีการศึกษาอย่างทั่วถึง ทั้งข้อมูลทางระบาดวิทยา และข้อมูลความแตกต่างของปัจจัยโน้มนำของแหล่งการติดเชื้อในประเทศไทย และรวมถึงปัจจัยทั่วไปตามลักษณะปรากฏภายนอก นอกจากนี้ในปัจจุบันมีการพบเชื้อชนิดอื่นที่มียังไม่เคยมีรายงานการตรวจพบในสุนัข จากตัวอย่างในระบบทางเดินหายใจของสุนัขที่แสดงอาการทางระบบทางเดินหายใจ ด้วยเหตุจูงใจนี้จึงนำมาถึงการคิดค้นและพัฒนาเทคนิคปฏิกิริยาลูกโซ่โพลีเมอเรสแบบมัลติเพล็กซ์ (multiplex PCR) เพื่อใช้ในการตรวจหาไวรัสพื้นฐานในการก่อโรคในกลุ่มอาการโรคติดเชื้อในระบบทางเดินหายใจสุนัขแบบซับซ้อน วิเคราะห์ถึงปัจจัยโน้มนำต่างๆอันมีผลต่อการติดเชื้อ และตรวจหาเชื้อชนิดอื่นที่อาจมีผลต่อการก่อโรคในระบบทางเดินหายใจแบบซับซ้อนในสุนัข ทั้งนี้การพัฒนาเทคนิคดังกล่าว ได้มีการทดสอบความจำเพาะและความไว เปรียบเทียบกับเทคนิคปฏิกิริยาลูกโซ่โพลีเมอเรสแบบปกติ และชุดตรวจที่มีจำหน่ายในท้องตลาด นอกจากนี้เทคนิคที่ถูกพัฒนาขึ้น ได้นำไปใช้ในการตรวจหาเชื้อไวรัสในตัวอย่างสารคัดหลั่งจากระบบทางเดินหายใจ จำนวน ๔๑๘ ตัวอย่าง จากสุนัขที่ป่วยด้วยโรคระบบทางเดินหายใจจำนวน ๒๐๙ ตัว โดยแบ่งแยกออกเป็นสุนัขที่ติดเชื้อจากชุมชนจำนวน ๑๓๓ ตัว และ สุนัขที่ติดเชื้อในโรงพยาบาลสัตว์จำนวน ๗๖ ตัว โดยมีการเก็บข้อมูลพื้นฐาน รวมถึงอาการทางคลินิกและประวัติของการทำวัคซีนของสุนัขแต่ละตัว หากตัวอย่างที่ให้ผลลบกับการตรวจด้วยเทคนิคดังกล่าว หรือเป็นตัวอย่างที่น่าสนใจ จะถูกนำมาศึกษาถึงนิเวศวิทยาของเชื้ออื่นในตัวอย่างดังกล่าว โดยการใช้เทคนิค Next Generation Sequencing (NGS)

จากการศึกษาพบว่า เทคนิคที่พัฒนา มีความไวรับมากกว่าร้อยละ ๘๗ และให้ความจำเพาะมากถึงร้อยละ ๑๐๐ เมื่อเทียบกับการตรวจด้วยเทคนิคปกติ จากการศึกษานี้ พบว่าสามารถตรวจพบเชื้อไวรัสพื้นฐานได้ทั้ง ๖ ชนิดจากทั้ง ๒ กลุ่มประชากร นอกจากนี้ยังพบว่าร้อยละ ๘๑.๒ และ ๗๘.๙ เป็นการติดเชื้อมากร่วมมากกว่าหนึ่งชนิด ในกลุ่มที่ติดเชื้อในสิ่งแวดล้อมและโรงพยาบาลตามลำดับ เชื้อ CIV และ CRCoV เป็นเชื้อหลักในการพบมากที่สุด ในขณะที่เชื้อไข้หัดสุนัข (CDV) มักพบในกลุ่มของสุนัขที่มีการติดเชื้อจากชุมชนมากกว่ากลุ่มของสุนัขที่มีการติดเชื้อในโรงพยาบาลสัตว์ ในส่วนของการวิเคราะห์ปัจจัยอื่นพบเพียงแค่อายุมีความสัมพันธ์กับอาการความรุนแรงของโรคเท่านั้น จากการศึกษานี้ยังสามารถตรวจพบเชื้อโบค่าไวรัสสุนัข ชนิดที่ ๒ (canine bocavirus type 2; CBoV-2) และ เชื้อเซอโครไวรัสสุนัข (canine circovirus; CanineCV) จากตัวอย่างที่ให้ผลลบจากการตรวจด้วยเทคนิคปฏิกิริยาลูกโซ่โพลีเมอเรสชนิดมัลติเพล็กซ์ จากการศึกษาทางลักษณะลำดับสารพันธุกรรมของเชื้อทั้ง ๒ ชนิด พบว่าเชื้อดังกล่าวเป็นสายพันธุ์ใหม่ ที่มีการเกิดการรวมกัน (recombination) ของสารพันธุ์กรรมของเชื้อไวรัสในกลุ่มเดียวกันแต่สายพันธุ์อื่น จากการศึกษาด้วยการตรวจหาสารพันธุกรรมของเชื้อไวรัสดังกล่าวด้วยวิธีการอื่น ทั้งการใช้ปฏิกิริยาลูกโซ่โพลีเมอเรสชนิดต่างๆและเทคนิคอินไซตู ไฮบริไดเซชั่น (*in situ* hybridization; ISH) ยังพบถึงการกระจายตัวของสารพันธุกรรมของไวรัสในอวัยวะอื่นๆ ซึ่งบ่งถึงการกระจายตัวของไวรัสผ่านทางระบบไหลเวียนโลหิต การศึกษาด้านจุลพยาธิวิทยาและการตรวจหารูปร่างของไวรัสด้วยกล้องจุลทรรศน์อิเล็กตรอน แสดงให้เห็นถึงไวรัสสายพันธุ์ใหม่นี้ มีความแตกต่างในการก่อโรคและกลไกการก่อพยาธิสภาพเมื่อเทียบกับสายพันธุ์อื่น การศึกษานี้ยังนำมาซึ่งข้อมูลทางระบาดวิทยาของเชื้อไวรัสพื้นฐานของกลุ่มอาการโรคติดเชื้อในระบบทางเดินหายใจสุนัขแบบซับซ้อน และไวรัสกลุ่มใหม่ที่อาจเป็นเชื้อในการก่อโรคในระบบทางเดินหายใจแบบซับซ้อนที่มีการระบาดในประเทศไทย รวมถึงปัจจัยโน้มนำต่างๆที่อาจส่งผลต่อการติดเชื้อ นำมาซึ่งผลประโยชน์และเป็นข้อมูลพื้นฐานในการศึกษาต่อไปในอนาคต

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KEYWORDS: CANINE INFECTIOUS RESPIRATORY DISEASE COMPLEX / CIRDC / RESPIRATORY / VIRUS / PCR / MULTIPLEX PCR

CHUTCHAI PIEWBANG: DEVELOPMENT OF MULTIPLEX RT-PCR AND METAGENOMIC ANALYSES OF CANINE INFECTIOUS RESPIRATORY DISEASE COMPLEX ASSOCIATED VIRUSES IN DOGS. ADVISOR: ASSOC. PROF. DR. SOMPORN TECHANGAMSUWAN, D.V.M., M.Sc., Ph.D., DTBVP., CO-ADVISOR: ASSOC. PROF. DR. ANUDEP RUNGSIPIPAT, D.V.M., Ph.D., DTBVP., PROF. YONG POOVORAWAN, M.D., 185 pp.

Canine infectious respiratory disease complex (CIRDC) viruses have been detected in dogs with respiratory illness. The CIRDC is associated with multiple factors depending on host susceptibility, environment and pathogens. Canine influenza virus (CIV), canine parainfluenza virus (CPIV), canine distemper virus (CDV), canine respiratory coronavirus (CRCoV), canine adenovirus type 2 (CAdV-2) and canine herpesvirus 1 (CaHV-1), are all associated with the CIRDC. Environmental factors serve as the one key point for infection. The transmission route can be via community-acquired infection (CAI) or hospital-associated infection (HAI), but the variable factors within these two routes are not well described. Moreover, novel pathogens that could not be identified, were detected from dogs showing respiratory problem. Thus, to allow diagnosis, establish the CIRDC viruses with associated risk factors for infection and discover the other novel pathogen causing respiratory disease in dogs, two conventional multiplex polymerase chain reactions (PCR) were developed to simultaneously identify four RNA and two DNA viruses associated with CIRDC. The developed multiplex PCRs were tested by sensitivity and specificity determination in comparison to conventional simplex PCR and a rapid three-antigen test kit. The two multiplex PCR assays were then validated on 418 respiratory samples collecting from 209 respiratory illness dogs locating in Thailand. In term of analysis of possible risk factors of CIRDC, the sample population of 209 dogs were divided in 133 community acquired infection (CAI) and 76 Hospital associated infection (HAI) groups. Essential signalments, clinical signs, vaccination status, and route of transmission were recorded for further analysis. Either negative-multiplex PCRs or interesting samples were subjected to further investigation by metagenomic analysis using Next Generation Sequencing (NGS) technology. Here, we established the developed multiplex PCR assays, which had a > 87% sensitivity and 100% specificity compared to their simplex counterpart. Compared to the three-antigen test kit, the multiplex PCR assays yielded 100% sensitivity and more than 83% specificity for detection of CAdV-2 and CDV, but not for CIV. Interestingly, all common six viruses were detected in both groups with CIV and CRCoV being predominantly found. Only CDV was significantly more prevalent in CAI than HAI dogs but not for the others. Multiple infections were found in 81.2% and 78.9% of CAI and HAI, respectively. Co-detection of CIV and CRCoV was significantly associated among study groups. Moreover, the clinical severity level was notably related to age of infected dogs, neither vaccination status, sex nor transmission route were noted. Surprisingly, a novel strain of canine bocavirus type 2 (CBoV-2) and canine circovirus (CanineCV) were identified in the samples from dogs that died in Thailand from acute disease, for which no causal etiological pathogen could be identified in routine screening assays. These novel CBoV-2 and CanineCV strains were named as CBOV TH-2016 and CanineCV TH/2016, respectively. Analysis of the complete coding sequences of CBoV TH-2016 and CanineCV TH/2016 showed that these viruses showed evidence of genetic recombination among other published strains by using similarity plot, bootscanning and cocktailed of statistical maximum likelihood algorithms. Use of both conventional or quantitative PCR and *in situ* hybridization showed the presence of these viruses in several tissues, suggesting hematogenous virus spread. Histopathological lesions associated with these viral infections in several organs and confirming of presence of the CBoV-2 infection using transmission electron microscope provided novel insights in the pathogenicity of canine bocavirus and canine circovirus infections and suggests that a novel recombinant of those viruses, which was discovered from deceased dogs showing negative for routine tests, may result in atypical syndrome.

Department:	Veterinary Pathology	Student's Signature	.....
Field of Study:	Veterinary Pathobiology	Advisor's Signature	.....
Academic Year:	2017	Co-Advisor's Signature	.....
		Co-Advisor's Signature	.....

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Abbreviations

AHDs:	Acute hemorrhagic diarrhea syndrome
BFDV:	Beak and feather disease virus
BHV:	Bovine herpesvirus
CA:	Community acquired
CaCV:	Canary circovirus
CAdV-2:	Canine adenovirus type 2
CaHV-1:	Canine hervesvirus -1
CAI:	Community acquired infection
CanineCV:	Canine circovirus
CBoV:	Canine Bocavirus
CCoV:	Canine Coronavirus (Pantropic canine coronavirus)
cDNA:	Complementary DNA
CDV:	Canine distemper virus
CIRDC:	Canine infectious respiratory disease complex
CIRD:	Canine infectious respiratory disease
CIV:	Canine influenza virus
CnMV:	Canine minute virus
CnPnV:	Canine pneumovirus
CPIV:	Canine parainfluenza virus
CPV:	Canine parvovirus
CRCoV:	Canine respiratory coronavirus
DIG:	Digoxigenin
DogCV:	Canine circovirus
EHV:	Equine herpesvirus
FBoV:	Feline bocavirus
FHV:	Feline herpesvirus
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
HA:	Hospital associate

HAI:	Hospital associated infection
HBoV:	Human bocavirus
HCoV:	Human coronavirus
HCV:	Human hepatitis C virus
HIV:	Human immunodeficiency virus
HN:	Hemagglutinin-neuraminidase
INIB:	Intranuclear inclusion body
ISH:	*In situ* hybridization
ITB:	Infectious tracheobronchitis
MDCK:	Madin-Darby canine kidney
MERs-CoV:	Middle East respiratory syndrome coronavirus
mPCR:	Multiplex polymerase chain reaction
mRT-PCR:	Multiplex reverse transcription polymerase chain reaction
NGS:	Next generation sequencing
NPHV:	Nonprimate hepacivirus
NPV:	Negative predictive value
NS:	Nasal swab
ORF:	Open reading frame
OS:	Oropharyngeal swab
PAstVs:	Porcine astroviruses
PBS:	Phosphate buffer saline
PCV:	Porcine circovirus
PBoV:	Porcine bocavirus
PhHV:	Phocid herpesvirus
PoSCV:	Porcine stool-associated circular virus
PPV:	Positive predictive value
qPCR:	Real-time polymerase chain reaction
SARs:	Severe acute respiratory syndrome
SH:	Small hydrophobic
SNPs:	Single nucleotide polymorphisms
TCID:	Tissue culture infective dose

TEM: Transmission electron microscope

TTHV: Bottlenose dolphin herpesvirus

CHAPTER 1

INTRODUCTION

1.1 The relation between all manuscripts in the thesis

Canine infectious respiratory disease complex (CIRDC) viruses have been detected in dogs with respiratory illness. Canine influenza virus (CIV), canine parainfluenza virus (CPIV), canine distemper virus (CDV), canine respiratory coronavirus (CRCoV), canine adenovirus type 2 (CAdV-2) and canine herpesvirus 1 (CaHV-1), are all associated with the CIRDC. To achieve diagnosis, two conventional multiplex polymerase chain reactions (mPCR) were developed to simultaneously identify four RNA and two DNA viruses associated with CIRDC that revealed on "Development and application of multiplex PCR assays for detection of virus-induced respiratory disease complex in dogs". The two mPCR assays were then validated on 102 respiratory samples collected from 51 dogs with respiratory illness by sensitivity and specificity determination in comparison to conventional simplex PCR and a rapid three-antigen test kit. All six viruses were detected in either individual or multiple infections. The developed mPCR assays had a more than 87% sensitivity and 100% specificity compared to their simplex counterpart. Compared to the three-antigen test kit, the mPCR assays yielded 100% sensitivity and more than 83% specificity for detection of CAdV-2 and CDV, but not for CIV. Therefore, the developed mPCR modalities were able to simultaneously diagnose a panel of CIRDC viruses and facilitated specimen collection through nasal (NS) or oropharyngeal (OS) swabs. This study also gave information of CaHV-1 in acute respiratory distress dogs in Thailand that was reported in "Viral molecular and pathological investigations of Canid herpesvirus 1 infection associated respiratory disease and acute dead in dogs". The CIRDC is commonly associated with multiple factors. The transmission route can be via community-acquired infection (CAI) or hospital-associated infection (HAI), but the variable factors within these two routes are not well described. The cross-sectional prevalence of all six CIRDC viruses detected in respiratory-disease dogs was investigated using developed mPCR assays as well as the

possibly related risk factors were analyzed. The study was revealed on “Cross-sectional investigation and risk factor analysis of community-acquired and hospital-associated canine infectious respiratory disease complex”. Two hundred and nine ill dogs consisting of 133 CAI and 76 HAI dogs were sampled through NS and OS separately. Signalments, clinical signs, vaccination status, and route of transmission were recorded for further analysis. All six viruses were detected in both groups with CIV and CRCoV being predominantly found. Only CDV was significantly more prevalent in CAI than HAI dogs but not for the others. Multiple infections were found in 81.2% and 78.9% of CAI and HAI, respectively. Co-detection of CIV and CRCoV was significantly associated among study groups. Moreover, the clinical severity level was notably related to age of infected dogs; neither vaccination status, sex nor transmission route was noted. Furthermore, novel canine bocavirus type 2 (CBoV-2) and canine circovirus (CanineCV) were discovered from dogs showing respiratory problem and yielding negative result for CIRDC screening, by using next generation sequencing (NGS) and metagenomic analysis. This investigation was shown in “A novel strain of canine bocavirus type-2 infection associated with intestinal lesions reminiscent of canine bocavirus type-1 infection” and “Genomic analysis of novel canine circovirus strains from Thailand: evidence for natural genetic recombination.” Also, phylogenetic analysis revealed that the novel CBoV-2 strain discovered in Thailand has genetically closed to the CBoV-2 strains isolated from South Korea and Hong Kong. The discovered CBoV-2 also exhibited the pathological lesions reminiscent to CBoV-1 infection, confirming by *in situ* hybridization. Although the CanineCV strains in Thailand are closely related to CanineCV strains discovered from American dog sera, they showed significantly different from those strains by pairwise identity matrix analysis. Possible genetic recombination conducted by different clades of phylogenetic tree, constructed by individual CBoV-2 and CanineCV strains discovering worldwide. Here, this study documented the novel CBoV-2 and CanineCV are recently circulating in Thailand. Further study of an association of disease in dogs should be taken into account.

All of manuscripts in partial fulfillment of the requirements for the degree of Doctor of Philosophy program in Veterinary Pathobiology.

1.2 Importance and rationale

Canine infectious respiratory disease (CIRD) is a complex disease occurring in dogs, affecting the larynx, trachea, bronchi, and occasionally the nasal mucosa (Buonavoglia and Martella, 2007). Kennel cough or infectious tracheobronchitis (ITB) is the term used to describe highly acute respiratory disease in dogs. Recently, either kennel cough, ITB or CIRD is usually used interchangeably for canine infectious respiratory disease complex (CIRDC) (Weese and Stull, 2013). The CIRDC is not only associated with germs, but also with environmental factors and host immune responses that playing an equally important role in this complex disease (Erles et al., 2004). The pathogens causing CIRDC are consisted of either viruses, bacteria or both, which transmit by aerosol from infected dogs particularly living in poor ventilation kennels, animal shelters and veterinary hospitals (Buonavoglia and Martella, 2007). The clinical manifestation is various ranging from asymptomatic clinical sign to fatal lung infection. Mild dry hacking cough and nasal discharge are predominately displayed as common clinical signs which disappear within a short period in most infected dogs. However, some hosts may develop a severe bronchopneumonia and immunosuppression which become fatal (Erles et al., 2004). Several natural outbreaks of the disease have shown that the etiology is complex and involves with a variety of viruses and bacteria. Viral pathogens associated with CIRDC include canine parainfluenza virus (CPIV) (Appel and Percy, 1970; Posuwan et al., 2010), canine adenovirus type 2 (CAdV-2) (Ditchfield et al., 1962; Binn et al., 1967; Wright et al., 1972; Erles et al., 2004; Posuwan et al., 2010), canine distemper virus (CDV) (Posuwan et al., 2010; Radtanakatikanon et al., 2013), canine herpes virus (CaHV) (Erles et al., 2004; Buonavoglia and Martella, 2007), canine influenza virus (CIV) (Buonavoglia and Martella, 2007; Payungporn et al., 2008; Posuwan et al., 2010) and canine respiratory coronavirus (CRCoV) (Erles et al., 2003; Ellis et al., 2005; Erles and Brownlie, 2005; Buonavoglia and Martella, 2007; Decaro et al., 2007).

Canine parainfluenza virus (CPIV) is a highly contagious paramyxovirus causing coughing, nasal discharge and fever when infected (Posuwan et al., 2010). Simultaneous infection with other viruses or secondary bacterial attack such as *Bordetella bronchiseptica* always promotes worse clinical symptoms (Binn et al., 1979;

Ueland, 1990). The parainfluenza virus is genetically similar to the simian virus 5 (SV5), which have been detected in humans occasionally although the relationship to any human disease remains contentious (Randall et al., 1987; Chatziandreou et al., 2004).

Canine adenovirus type 2 (CAdV-2) is more frequently associated with the CIRDC. The CAdV-2 infection usually occurs in puppies but sporadically detected in adult dogs (Buonavoglia and Martella, 2007). The host range of the virus is broad including a number of mammalian species such as foxes (Garcelon et al., 1992; Robinson et al., 2005), black bears, fishers, polar bears, wolves (Philippa et al., 2004) and Steller sea lions (Burek et al., 2005). Thus, wild-life animals might be a potential source of infection for domestic dogs (Buonavoglia and Martella, 2007).

Canine distemper virus (CDV) is one of highly contagious CIRDC viruses and belongs to morbillivirus, family *Paramyxoviridae* (Radtanakatikanon et al., 2013). The CDV is usually associated with severe respiratory symptoms and accompanied by systemic lesions such as gastrointestinal, integumentary, and central nervous systems (Erles et al., 2004; Carvalho et al., 2012; Radtanakatikanon et al., 2013; Pratakpiriya et al., 2017). Interestingly, CDV-infected dogs are considered as an animal model for spontaneous human demyelination which is analogous to multiple sclerosis (Beineke et al., 2009; Wyss-Fluehmann et al., 2010).

Canine herpes virus type 1 (CaHV-1) is *Alphaherpes* virus. The CaHV-1 has been isolated from dogs with CIRDC, but its pathogenic role remains uncertain (Binn et al., 1979; Erles et al., 2004). The CaHV-1 infected puppies show fatal generalized necrotizing and hemorrhagic lesions. The CaHV-1 is persisted and it becomes to latent infection; however, the virus can be reactivated when the dogs are under stress and immunosuppressive (Okuda et al., 1993; Miyoshi et al., 1999).

Canine influenza virus (CIV) has been isolated from dogs since 2004 and almost dogs that are infected with CIV show respiratory illness ranging from mild to severe symptoms (Crawford et al., 2005; Yoon et al., 2005; Payungporn et al., 2008). The virus belongs to influenza A virus, H3N8, which is genetically related with equine influenza virus (Crawford et al., 2005). Moreover, the CIV subtype H3N2 is originated from avian influenza subtype H3N2 as well(Song et al., 2008). These findings also explained that emerged CIVs are consistent with a single interspecies virus transfer. Thus, the virus

likely got adapted to the dogs by accumulation of point mutations rather than genetic reassortment with other strains of influenza A virus (Crawford et al., 2005). Such a scenario provokes a public health concern that may raise possibility of the emergence of the new recombinant companion canine and feline influenza viruses. Therefore, zoonotic potential cannot be excluded (Song et al., 2011).

Recently, canine respiratory coronavirus (CRCoV) is usually detected in kennels and mostly associated with mild respiratory symptoms (Erles et al., 2003; Erles et al., 2004; Buonavoglia and Martella, 2007). The CRCoV is genetically closed to human coronavirus OC43 (HCOV-OC43) and bovine coronavirus. This finding suggests a recent common ancestor of these viruses and demonstrates the occurrence of repeated host-species shifts (Erles et al., 2003). The emerged CRCoV replicates in respiratory epithelium, leading to secondary bacterial or other viral infection (Buonavoglia and Martella, 2007).

Diagnosis of CIRDC virus becomes important for appropriate treatment plan, prognosis and preventive strategies. Various diagnostic tests are available for these infections; however, many are not practical due to long processing time, poor specificity or sensitivity, and costly diagnostic tools (Posuwan et al., 2010). Some of these methods include viral culture and isolation technique. Such modalities cannot be performed in all viruses due to poor ability of virus growth in cell culture. Moreover, evaluation of serum antibody titer is usually requiring convalescent testing weeks later to identify a rise in the titer. Thus, the rapid method for detection of CIRDC viruses is in need (Payungporn et al., 2006). Genetic molecular technique is an appropriate method to detect these viruses. Multiplex polymerase chain reaction (mPCR) technique has been used to diagnose multiple pathogens (Payungporn et al., 2006; Thontiravong et al., 2007). In addition, it shortens the processing time allowing rapid diagnosis and quick implementation of appropriate treatment and isolation precautions when necessary. The mPCR also provides acceptable sensitive and specific test results, serving a screening method. Furthermore, similarity of clinical signs in different viral infections and a lack of the CIRDC prevalence in dogs in Thailand may contribute confusion for tentative primary etiology. Thus, clinical signs or lesion relationship among virus infection and information of the prevalence of CIRDC infected

dogs must be intensively studied. Even though most of viruses that cause of CIRDC can be controlled by vaccine, suffering dogs from respiratory infections remain increasingly (Erles et al., 2004; Posuwan et al., 2010). Thus, improper vaccination and/or the viruses may adapt its virulence, might play roles in the increasing number of CIRDC affected dogs.

Meanwhile many novel viruses are discovered recently, the emerging viruses have been reported continuously such as Human immunodeficiency virus (HIV) (Hirsch et al., 1995), Ebola virus (Pourrut et al., 2005), SARs virus (Chan et al., 2003), avian influenza A H5N1 (Payungporn et al., 2006) and a variety of hepatitis viruses such as Non-primate hepacivirus (NPHV), which is genetically closed to human hepatitis C virus (HCV) (Kapoor et al., 2011; Lyons et al., 2012). Hepatitis E virus is discovered from pigs and causes hepatic infection in humans as well (Meng et al., 1997). Thus, many viruses in animals play roles in zoonotic potential. Moreover, some viruses may increasingly turn virulence of infectivity and expand to new hosts. Therefore, monitoring of emerging viruses is important. Many viruses were discovered since genetic researches and its applications were famous. Recently, pyrosequencing has been developed as alternative sequencing method (Ronaghi, 2001). This technique has been widely performed to approach for the characterization of nucleic acid in deep details, potentially providing advantages of accuracy, flexibility, parallel processing and can be easily automated. In addition, the pyrosequencing has been used for both *de novo* sequencing and confirmatory sequencing for metagenomic analyses successfully (Ronaghi, 2001; Fakruddin et al., 2012). Viral metagenomics avoid the potential limitations of traditional methods, including the failure of virus to replicate in cell cultures, unsuccessful PCR amplification or microarray hybridization due to high-level genetic divergence from known viruses, and the failure of antibodies binding to known viruses or producing the cross-reaction. The application of viral metagenomics may also be useful for the study of diseases with unexplained etiologies possibly involving uncharacterized viruses or combined viral infections. Thus, the next generation sequencing and metagenomic analysis have been mostly used to discover novel viruses (Handelsman, 2004).

Despite the fact that viruses can adapt and infect cross-species of hosts, both genetic sequencing and phylogenetic analysis are useful applications to analyze and provide genetic relationship among subtypes of the viruses. These methods can follow and quarantine new emerging viruses, which may adapt to higher infectivity. The CIRDC dog prevalence in Thailand is still questioned. Alternatively, most viruses that cause CIRDC in dogs are genetically similar to viruses that cause respiratory disease in humans as well. Thus, this study was subdivided into 3 parts; (1) Development of multiplex reverse transcription polymerase chain reaction (mRT-PCR) for virus detection in canine infectious respiratory disease complex (CIRDC); (2) Simultaneous viral detection of canine infectious respiratory disease complex (CIRDC) by a multiplex reverse transcription polymerase chain reaction (mRT-PCR) assay with clinical and pathological changes in respiratory disease dogs and (3) The metagenomic analyses of viruses isolated from respiratory distress in dogs with unknown causes.

1.3 Literature review

Canine infectious respiratory disease complex (CIRDC) or canine infectious tracheobronchitis (ITB) has been known as kennel cough, is a highly contagious respiratory disease in dogs that is transmitted by aerosol from infected dogs (Buonavoglia and Martella, 2007). The disease is characterized by dry coughing, which is cleared within short times in most cases; however, some dogs become fatal when severe bronchopneumonia is developed (Erles et al., 2004). The CIRDC is a very common disease complex caused by many different viruses and bacteria. Moreover, stress, overcrowded animals and poor ventilation in kennels, animal shelters and veterinary hospitals are prone to be the risk of infections (Erles et al., 2004; Weese and Stull, 2013). Several studies revealed the disease is a complex, which is caused by variety of pathogens, especially viruses. The viruses that have been recognized as pathogen of CIRDC are consisted of canine parainfluenza virus (CPIV) (Appel and Percy, 1970; Posuwan et al., 2010), canine adenovirus type 2 (CAdV-2) (Ditchfield et al., 1962; Binn et al., 1967; Wright et al., 1972; Erles et al., 2004; Posuwan et al., 2010), canine distemper virus (CDV) (Posuwan et al., 2010; Radtanakatikanon et al., 2013), canine herpes virus 1 (CaHV-1) (Erles et al., 2004; Buonavoglia and Martella, 2007), canine influenza virus (CIV) (Buonavoglia and Martella, 2007; Payungporn et al., 2008; Posuwan et al., 2010) and canine respiratory coronavirus (CRCoV) (Erles et al., 2003; Ellis et al., 2005; Erles and Brownlie, 2005; Buonavoglia and Martella, 2007; Decaro et al., 2007).

Viral information

Canine parainfluenzavirus (CPIV)

Canine parainfluenza virus is belonging to family *Paramyxoviridae* that is composed of a single stranded RNA genome of negative polarity, surrounded by a lipid envelope of host cell origin (Lamb and Kolakofsky, 2001). There are 7 genes which encode 8 proteins: nucleoprotein (N), V/phosphoprotein (V/P), matrix (M), fusion (F), small hydrophobic (SH), hemagglutinin-neuraminidase (HN) and large (L) genes. The HN protein is involved in cellular attachment to initiate virus infection. The F protein can mediate fusion of viral envelope with cell membrane (Buonavoglia and Martella, 2007).

The CPIV is genetically similar to simian virus 5, which had been isolated from humans, suggesting the closed relationship among two viruses (Randall et al., 1987; Chatziandreou et al., 2004). The CPIV mostly replicates in epithelial-lining cells of nasal mucosa, pharynx, trachea and bronchi. Thus, CPIV infection is usually restricted to upper respiratory tract; however, CPIV could be isolated from local respiratory lymph nodes (Binn et al., 1979; Buonavoglia and Martella, 2007). In addition, the virus also promotes *Bordetella bronchiseptica* infection in respiratory tract (Binn et al., 1979; Ueland, 1990). The CPIV infected dogs usually show catarrhal rhinitis and tracheitis with mononuclear and polymorphonuclear inflammatory cell infiltration into mucosal and submucosal layers of respiratory tract (Appel and Percy, 1970).

Canine adenovirus type 2 (CAdV-2)

Canine adenovirus (CAdV), a double stranded DNA virus, is belonged to *Adenoviridae* family. CAdV is subdivided into two types; CAdV-1 and CAdV-2, with different target cells (Buonavoglia and Martella, 2007). Vascular endothelial cells, liver and lung parenchyma serve as target cells of CAdV-1, while respiratory tract epithelium is target cells of CAdV-2 (Buonavoglia and Martella, 2007; Chvala et al., 2007). Moreover, intestinal epithelium can be infected with CAdV-2 in limited degree (Chvala et al., 2007). Oro-nasal transmission is a route of infection for CAdV-2. The virus replicates in respiratory epithelium and mucosal epithelium of pharynx as well as tonsillar crypts and type II pneumocyte. Respiratory signs are shown with varying degree. Some dogs can develop bronchiolitis obliterans due to type II pneumocyte infection (Buonavoglia and Martella, 2007). The host range of CAdV-2 includes a large number of mammalian animals. Thus, wild-life mammalians may play a role in CAdV-2 infection (Buonavoglia and Martella, 2007).

Canine distemper virus (CDV)

Canine distemper virus (CDV), a single-stranded negative-sense RNA virus, belongs to *Paramyxoviridae* family. The virus composes of six protein genes; the nucleocapsid (N), phosphoprotein (P), large (L) protein as a helical nucleocapsid

core, the hemagglutinin (H) glycoprotein, and the fusion (F) protein as an enveloped protein and matrix (M) protein is assembling between the envelope and nucleocapsid core. P protein that acts as a co-factor is the most conserved structure among the *Morbillivirus*. The F protein mediates the fusion of the viral and cellular membrane which enables the entry of the viral genome into host cells (von Messling et al., 2001) and play a key in determinant of persistent infection (Plattet et al., 2005). H protein is responsible for viral attachment to the host's cell and fusogenic activity, and may also play a role in host's humoral immune response. Thus, the F and H proteins are the most variable parts of virus and have often been used to assess genetic changes between CDV isolates (Pardo et al., 2005; Lee et al., 2010; Radtanakatikanon et al., 2013). Lymphocytes and epithelial cells serve as a cellular tropism of CDV after dogs are infected by aerosol (Carvalho et al., 2012). Therefore, respiratory tract, gastrointestinal tract, integumentary system, lymphatic system, urinary bladder and central nervous system are affected by CDV infection (Erles et al., 2004; Carvalho et al., 2012; Radtanakatikanon et al., 2013).

Canine herpesvirus type 1 (CaHV-1)

Canine herpes virus (CaHV-1) is classified to *Alphaherpesviridae* family. The CaHV-1 appears to be a monotypic virus, which genetically similar to feline herpesvirus-1 (FHV-1), phocid herpesvirus-1 (PhHV-1) and equine herpesvirus-1 (EHV-1) and -4 (EHV-4) (Rota and Maes, 1990; Remond et al., 1996). However, the host range of CaHV-1 is restricted to dogs only. The viruses infect dogs by direct contact. Moreover, the virus can be transmitted transplacentally, leading to fatal disease in very young puppies and causing generalized necrotizing and hemorrhagic disease (Hashimoto et al., 1982). While adult infected dogs often show asymptomatic signs or mild rhinitis and pharyngitis (Carmichael et al., 1995). The virus becomes latent by persisting in trigerminal ganglia and reactivates by stress induction (Okuda et al., 1993; Miyoshi et al., 1999). Several studies revealed that co-infection of other viruses with CaHV-1 leads to severity. However, the role of CaHV-1 associated with CIRDC remains unclear (Binn et al., 1979; Erles et al., 2004).

Canine influenza virus (CIV)

Canine influenza virus is influenza A virus in family *Orthomyxoviridae*, which is a single stranded RNA virus and composed of eight segments (Fouchier et al., 2005). The CIV has been reported to infect and cause respiratory disease in dogs. Canine influenza subtype H3N8 had been reported in 2004 (Crawford et al., 2005). The virus emerged in Florida, USA by direct transmission from equine influenza subtype H3N8 to the greyhound sheltering dogs with signs of respiratory distress. Moreover, the virus spreads to 11 states throughout the USA rapidly (Yoon et al., 2005). Molecular and antigenic analyses proved that CIV is closely related to H3N8 equine influenza virus (Crawford et al., 2005; Yoon et al., 2005). Sequence and phylogenetic analysis of CIV genomes revealed that CIV forms a monophyletic group, consisting with a single interspecies virus transfer. The virus likely got adapted to canine host by accumulation of point mutations rather than by exchange of genome segments as genetic reassortment (Crawford et al., 2005). In addition, the CIV also emerged in Korea in 2007 and was different from CIV that emerged in USA. The CIV in Korea was originated from avian influenza subtype H3N2 (Song et al., 2008). In Thailand, there is a report of CIV infection in dogs during 2008-2009 with prevalence 2.94% in healthy dogs and 1.83% in respiratory-infected dogs (Posuwan et al., 2010). In addition, one influenza A was characterized as an avian-like influenza H3N2 in 2012 in a dog (Bunpapong et al., 2014). CIV infected dogs showed ranging clinical signs from mild to severe respiratory illness. Mild illness is described in most cases which show fever, cough and nasal discharge while severe signs are characterized by bronchopneumonia and hemorrhage in respiratory tract (Crawford et al., 2005; Yoon et al., 2005; Payungporn et al., 2008; Posuwan et al., 2010). Recently, the CIV can transmit from CIV-infected dogs to pet cats directly, suggesting that the virus may adapt or increase its infectivity. This finding raises public health concern for monitoring the new zoonotic disease (Song et al., 2011).

Canine respiratory coronavirus (CRCoV)

Canine respiratory coronavirus (CRCoV) belongs to *Coronaviridae* family. CRCoV is classified to coronavirus group 2 as well as bovine coronavirus and human coronavirus OC43 (Erles et al., 2003). The virus is a novel pathogen which detected in respiratory tract of dogs with varied respiratory symptoms ranging from mild to severe illness, leading to respiratory distress caused by the virus replication in respiratory epithelium (Chilvers et al., 2001; Erles et al., 2003). Thus, the CRCoV is counted for a causative agent of CIRDC (Erles et al., 2003). Recently, few CRCoV strains have been discovered in some countries such as UK (Priestnall et al., 2006), Japan (Yachi and Mochizuki, 2006), Italy (Decaro et al., 2007), and Korea (An et al., 2010a). The virus can be detected at any ages of dogs but the prevalence is higher in aged dogs that suffered from respiratory distress (Priestnall et al., 2006; Priestnall et al., 2009). The role of CRCoV associated with CIRDC is still unclear. The seroconversion is observed in naïve immune responses with clinical symptoms, suggesting CRCoV may likely play a role in CIRDC directly (Erles et al., 2003). According to CRCoV replication, damaging of respiratory epithelium leads to super-infection and colonization of other pathogens (Buonavoglia and Martella, 2007). Recently, a novel coronavirus which has been called Middle East Respiratory Syndrome Coronavirus (MERS-CoV) was firstly isolated from sputum of a patient with acute pneumonia in Saudi Arabia (Zaki et al., 2012). The MERs-CoV has spread rapidly as evidence of human infection in Saudi Arabia, Qatar and United Arab Emirates. Furthermore, a tourist in UK, France, Jordan and Malaysia who returning from middle-east countries got illness and transmitted the infection to at least one other person (de Groot et al., 2013). To date, the largest cluster of MERS-CoV infections has been reported to 23 confirmed cases with 15 of them are dead. Interestingly, the infected cases involved ICUs, general patients and hemodialysis cases. Thus, most of those cases were hospital acquired infection. A MERS-CoV reservoir has yet to be identified; however, the MERS-CoV has genetically similar to as-yet-unclassified viruses from insectivorous European and African bats (Annan et al., 2013; Cotten et al., 2013). Moreover, recent studies suggest that MERS-CoV has

been circulating among camels (Reusken et al., 2013) and proving that the camels serve as a reservoir hosts for MERS-CoV infection in humans (Azhar et al., 2014).

Diagnostic modules

Currently, virus isolation in tissue culture serves as the gold standard diagnostic method. This method is high accurate for virus detection even though it is laborious and time consuming. Moreover, the various serological tests are available for diagnosis of many infectious diseases; however, time consuming and defection of accuracy of current infection become disadvantage of this method. Polymerase chain reaction (PCR) technique has been used for detection of various infections due to more rapid, sensitive and specific than conventional methods. However, using simplex PCR technique for detection of several infectious pathogens is labor-intensive and expensive. Moreover, low amount of sample is not suitable for this assay. These limitations of simplex PCR can be overcome by applying a multiplex PCR (mPCR) assay. This method is significantly improved to cover the disadvantages of simplex PCR technique. The mPCR is achieved by incorporating of multiple primers, which simultaneously amplify several nucleic acids in a single reaction.

Numerous studies that using mPCR or multiplex reverse transcription PCR (mRT-PCR) technique for DNA or RNA viral detection in respiratory distress patients were documented including Influenza A, B, and C viruses, Respiratory syncytial virus, adenoviruses, human parainfluenza viruses, human coronaviruses, human enteroviruses and rhinoviruses (Coiras et al., 2003; Coiras et al., 2004). Moreover, typing and subtyping influenza viruses in humans are successfully performed by this assay (Payungporn et al., 2004). In veterinary medicine, this technique was rarely used to detect respiratory viruses; however, various target genes of H5N1 influenza A virus were detected by using single step multiplex real-time RT-PCR (Payungporn et al., 2006). Therefore, the H5 and H7 avian influenza A and B viruses have been effectively detected by using mRT-PCR (Thontiravong et al., 2007; Wu et al., 2008). Besides, the mPCR is easily adapted to conventional PCR, real-time PCR and nested PCR as well. Although, such technique has been known to detect various viruses simultaneously,

the CIRDC viruses are never detected by this technique in Thailand. Thus, the method will be further developed and hence, reported an even more rapid, specific and sensitive assay based on mRT- PCR using primers for specific respiratory viral pathogens.

Canine respiratory disease complex (CIRDC) is a disease complex which is pronounced from multifactorial etiologies including host susceptible, pathogens and environmental factors. Hospital-associated infections are an ever-present risk in veterinary health care facilities. Thus, there has been increasing monitor about these infections in recent years because of emergence and dissemination of multidrug resistant pathogens. Nearly half of CIRDC infected dogs may concern to classify the primary cause of infection from veterinary facilities, leading to superimpose of multiple pathogen results to sudden fatal disease (Weese and Stull, 2013). However, some CIRDC-infected dogs may have etiology of infection from endemic disease from community without any hospital visit. Thus, classifying of presumable etiology of infection might play an important role to CIRDC quarantines.

The prevalence of each CIRDC virus is varied on geographic distribution, seasons, sample sizes and sample collection site. Previous studies showed that CAdV-2, CIV, CDV, and CPIV were isolated from respiratory distress dogs in Thailand for 9.71%, 1.83%, 2.75% and 11.93%, respectively (Posuwan et al., 2010). However, 12.8% of CPIV infected dogs were detected in USA even though CAdV-2 was not detected (Erles et al., 2004). In addition, CPIV and CaHV-1 were isolated in dogs in USA that suffered from respiratory diseases in 19.4% and 12.8%, respectively (Erles et al., 2004). The novel CRCoV was also detected in UK (Erles et al., 2003), USA (Priestnall et al., 2006), Japan (Yachi and Mochizuki, 2006) and South Korea (An et al., 2010a). Recently, information of CRCoV infection in Thailand has been published that the coronavirus genetic materials were detected from respiratory samples in respiratory suffered dog using polymerase chain reaction (PCR) (Piewbang et al., 2016). This research also presumed that virus is the CRCoV infection. However, information of CaHV-1 infection in Thailand is still questioned. Recently, serology against some CIRDC viruses was documented. Antibodies against CRCoV are commonly detected in UK, Republication of Ireland, USA, Japan, Korea, Italy and New Zealand for 36.2% 30.3%, 54.7%, 17.8%, 12.8%, 32.1%and 29%, respectively (Erles et al., 2003; Kaneshima et al., 2006; Priestnall et al., 2006;

Decaro et al., 2007). Alternatively, the seropositive for CaHV-1 in house hold colony-bred dogs was 88%, 45.83%, 27.9% and 39.3% in England, Belgium, Italy and Netherlands, respectively (Reading and Field, 1999a; Rijsewijk et al., 1999; Ronsse et al., 2002). However, CIV serological prevalence was increased in pet dogs in Korea from 5.1% to 12.8% in 2007 and 2010 (Song et al., 2011) as well as 6.7% in China (Li et al., 2010).

Information of target cells in some CIRDC viruses has been published; however, some the viruses are still questioned. A canine adenovirus type 2 virus (CAdV-2) mostly replicates in non-ciliated bronchiolar epithelium, surface cells of nasal mucosa, tonsil, pharynx, trachea and its regional lymph nodes and type 2 alveolar epithelial cells. Thus, clinical signs of CAdV-2 infection might be consistent with damage of bronchial epithelial cells as evidence of bronchiolitis and bronchiolitis obliterans associated with interstitial pneumonia (Buonavoglia and Martella, 2007). Canine herpes virus (CaHV) often causes of fatal septicemic disease in puppies. Moreover, the virus can cause a fatal generalized necrotizing and hemorrhagic disease in various tissues; however, some infected dogs might show only upper respiratory distress (Hashimoto et al., 1982). Although canine influenza virus (CIV)-infected dogs revealed extensive hemorrhagic pneumonia and hemothorax. Infected lungs exhibited red to red-black discoloration with marked palpable firmness. Dogs with the CIV infection are histologically revealed inflammation of trachea and bronchus. Lung sections revealed severe hemorrhagic interstitial pneumonia with alveolar septal thickening. Atelectasis associated with pyogranulomatous bronchointerstitial pneumonia was observed in CIV infected dogs (Crawford et al., 2005; Yoon et al., 2005). In case of the canine parainfluenza virus (CPIV) infection, the infected dogs are mostly characterized by upper respiratory problems. Histologically, catarrhal rhinitis and tracheitis with mono- and poly-morphonuclear cells infiltrated in the mucosa and submucosa were observed. Infected bronchi may contain leukocytes and cellular debris (Buonavoglia and Martella, 2007). Canine distemper virus (CDV) infection usually causes fatal illness. Macroscopic finding in CDV infected dogs showed pneumonia associated with pulmonary edema and congestion. Interstitial pneumonia, syncytial formation of bronchiolar epithelium, proliferation of pulmonary alveolar macrophages and bronchiolar epithelium sloughing

have been noted in CDV infection (Radtanakatikanon et al., 2013). In contrast, the CRCoV infection may contribute the various pathology begins mild tracheitis with blunt cilia until severe hemorrhagic pneumonia (Buonavoglia and Martella, 2007).

Some human patients showed signs of infection, but any pathogens cannot be detected in these patients meanwhile many viruses were discovered as novel viruses recently. The emerging viruses have been reported continuously such as Human immunodeficiency virus (HIV) (Hirsch et al., 1995), Ebola virus (Pourrut et al., 2005), SARs virus and avian influenza H5N1 (Payungporn et al., 2006). These viruses are previously discovered from animals; however, they also being isolated from humans with diseases. Although HIV is found in monkeys and the SARS is found in various animals. This particular virus was completely new. It is rather difficult to predict what those are going to cause any disease. On the contrary, some causative viruses found in humans were also isolated from animals such as nonprimate hepacivirus (NPHV) (Kapoor et al., 2011; Lyons et al., 2012). The NPHV is genetically closed to human hepatitis C virus (HCV) and it has been isolated from dogs with respiratory illness and horse with normal clinical appearance. Hepatitis E virus is discovered from pigs and it causes hepatic infection in humans as well (Meng et al., 1997). Thus, many viruses in animals play roles in zoonotic potential. Moreover, some viruses may increasingly turn virulence of infectivity and expand their host tropism. Thus, monitoring of emerging viruses is important. Many viruses were discovered since genetic researches and its applications were famous. Currently, genetic sequencing is one of the most important platforms to study organisms in biological system, giving more knowledge of hidden or novel organisms (Cheung et al., 2013). The application of genetic sequencing has been used for grouping or classification of unknown organisms as well. Chain termination sequencing method, which is known as Sanger sequencing method, has been widely used for the study of genetic alignment of any organisms (Sanger et al., 1977). Although, the Sanger method is worldwide manipulated, there are limitations of this method has been recognized.

Recently, next generation sequencing (NGS) is adopted as an alternative sequencing method and widely performed to approach the nucleic acid characterization in deep details (Ronaghi, 2001). It potentially provides advantages of

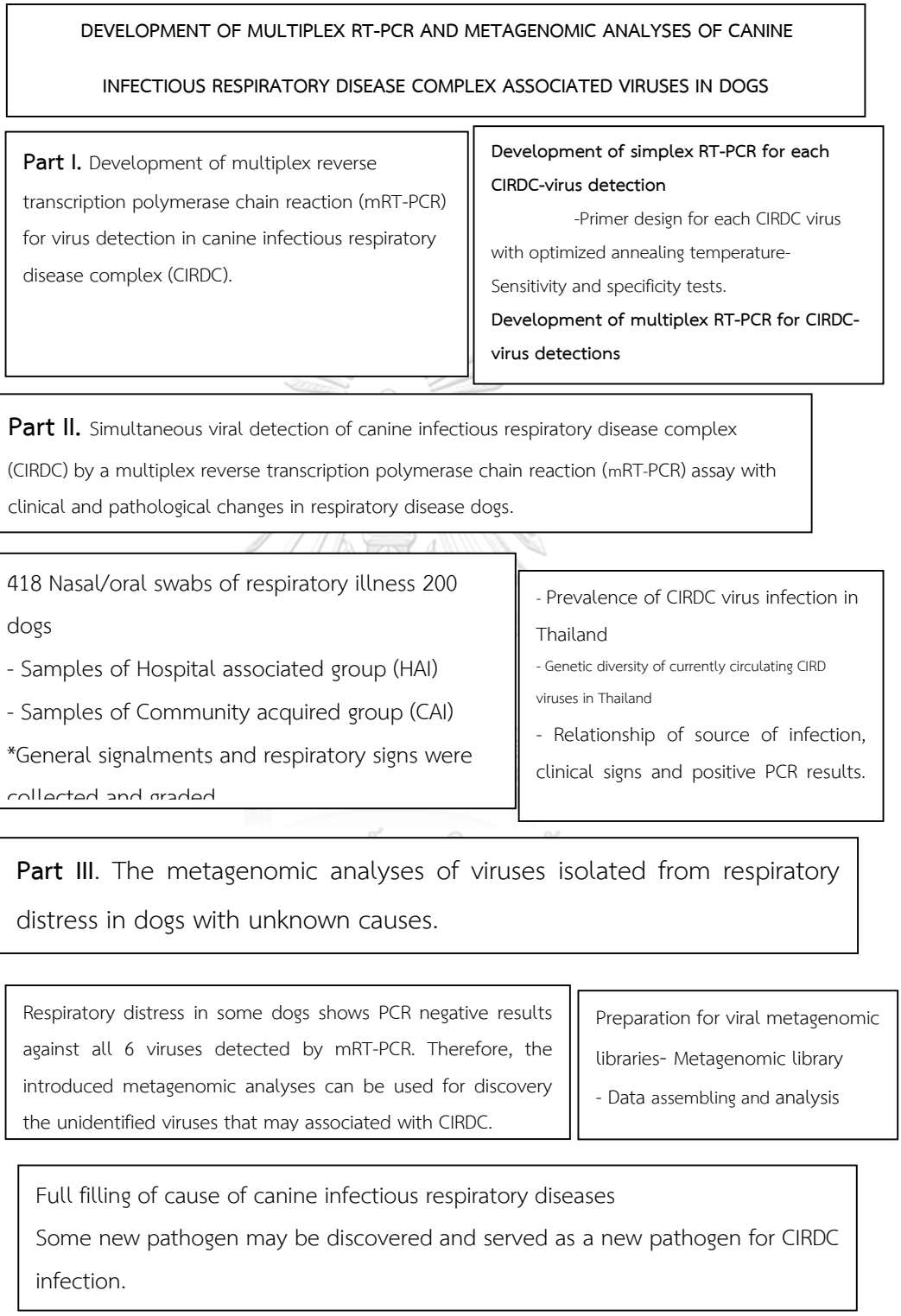
accuracy, flexibility, parallel processing and can be easily automated (Fakruddin et al., 2012). In addition, the NGS has been used for both *de novo* sequencing and confirmatory sequencing successfully (Fakruddin et al., 2012). Applications of NGS have been recognized for analysis of Single Nucleotide Polymorphisms (SNPs) as well as identification of various pathogens, especially viruses (Ahmadian et al., 2000a; Cheung et al., 2013). Moreover, the NGS has been demonstrated the ability to detect mutation and to determine the difficult secondary gene structures (Ronaghi et al., 1999; Ahmadian et al., 2000b). The NGS also provides the sequencing ability more than 1,000 bp, which is impossible to gain by conventional Sanger sequencing method (Fakruddin et al., 2012). Another highly significant application is whole genome analysis, preferring metagenomic library (Handelsman, 2004). Viral metagenomics avoids the potential limitations of traditional methods, including the failure of virus to replicate in cell cultures, unsuccessful PCR amplification or microarray hybridization due to high-level genetic divergence from known viruses, and the failure of antibodies to known viruses to cross-react (Victoria et al., 2009). The application of viral metagenomics may also be useful for the study of diseases with unexplained etiologies possibly involving uncharacterized viruses or combined viral infections. Thus, the NGS has been mostly used to discover novel viruses such as porcine stool-associated circular virus 2 and 3 (PoSCV2 and PoSCV3) (Cheung et al., 2013), porcine astroviruses (PAstVs) (Xiao et al., 2013), Lamivudine-resistant Hepatitis B virus (Lindstrom et al., 2004)and Canine hepatitis C virus in dogs (Kapoor et al., 2011).

Thus, this method provides more accuracy for genetic sequencing which is mostly modestly used for modern field research on virus discovery.

1.4 Objectives

- To develop the diagnostic tool for viral associated pathogens of CIRD by the simplex and multiplex reverse transcription polymerase chain reaction (mRT-PCR) with sensitivity and specificity comparison.
- To establish disease surveillance of each particular virus for epidemiological monitoring the potentially air-borne zoonoses from animals to humans.
- To investigate the prevalence and genetic diversity of currently circulating CIRD associated viruses in Thailand and compare with the previous known sequences deposited in GenBank.
- To discover the novel strain of virus or novel virus that can be or not associated with respiratory illness in dogs.
- To monitor the emerging virus which is possible to transmit to other animals and humans, alerting to public health concerns.

1.5 Conceptual framework



1.6 Advantages of the study

- Establishment the mRT-PCR assays as diagnostic tool for viral associated pathogens of CIRD with high sensitivity and specificity.
- Documentation the disease surveillance of each particular virus for further epidemiological monitoring and vaccine development.
- Computation the prevalence and genetic diversity of currently circulating CIRD associated viruses in Thailand and compare with the previous known sequences deposited in GenBank.
- Estimation the risk factors associated to CIRDC-virus infection, giving awareness information to veterinarians and pet owners.
- Establishment the novel strain of canine bocavirus 2 (CBoV-2) and canine circovirus (CanineCV), circulating in Thailand and giving information of such natural recombination events.

CHAPTER 2

2.1 DEVELOPMENT AND APPLICATION OF MULTIPLEX PCR ASSAYS FOR DETECTION OF VIRUS-INDUCED RESPIRATORY DISEASE COMPLEX IN DOGS

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ABSTRACT

Canine infectious respiratory disease complex (CIRDC) viruses have been detected in dogs with respiratory illness. Canine influenza virus (CIV), canine parainfluenza virus (CPIV), canine distemper virus (CDV), canine respiratory coronavirus (CRCoV), canine adenovirus type 2 (CAdV-2) and canine herpesvirus 1 (CaHV-1), are all associated with the CIRDC. To allow diagnosis, two conventional multiplex polymerase chain reactions (PCR) were developed to simultaneously identify four RNA and two DNA viruses associated with CIRDC. The two multiplex PCR assays were then validated on 102 respiratory samples collected from 51 dogs with respiratory illness by sensitivity and specificity determination in comparison to conventional simplex PCR and a rapid three-antigen test kit. All six viruses were detected in either individual or multiple infections. The developed multiplex PCR assays had a > 87% sensitivity and 100% specificity compared to their simplex counterpart. Compared to the three-antigen test kit, the multiplex PCR assays yielded 100% sensitivity and more than 83% specificity for detection of CAdV-2 and CDV, but not for CIV. Therefore, the developed multiplex PCR modalities were able to simultaneously diagnose a panel of CIRDC viruses and facilitated specimen collection through being suitable for use of nasal or oral samples.

KEYWORDS: CIRD, diagnosis, multiplex PCRs, Thailand

INTRODUCTION

Canine infectious respiratory disease complex (CIRDC), also known as kennel cough or infectious tracheobronchitis, is a highly acute respiratory disease in dogs that affects the larynx, trachea, bronchi and, occasionally, the nasal mucosa (Decaro et al., 2010; Weese and Stull, 2013). The CIRDC is not only associated with infectious pathogens, but environmental factors and host immune responses also play an equally important role (Erles et al., 2004). The pathogens causing CIRDC consist of viruses, bacteria or both, and are airborne-transmitted from infected dogs, particularly those living in poorly ventilated kennels, animal shelters and veterinary hospitals (Decaro et al., 2010). A CIRDC infection usually results in delaying of rehoming, interruption of training courses and requires high cost treatments (Erles et al., 2003). Mildly productive cough and nasal discharge initially present as the most common clinical signs, which is self-limited within a short period in most infected dogs. It is not fatal unless other complicating factors are involved, such as secondary bacterial infection or an immunosuppressed condition (Erles et al., 2004). Several episodes of CIRDC infection have been shown for a variety of viral agents. Canine parainfluenza virus (CPIV) is the most frequently detected agent in CIRDC dogs (Posuwan et al., 2010). Canine adenovirus type 2 (CAdV-2) and canine distemper virus (CDV) have also frequently been reported in dogs with severe respiratory distress (Erles et al., 2004; Posuwan et al., 2010; Radtanakatikanon et al., 2013). Canine herpesvirus 1 (CaHV-1) has been isolated from both puppies and adult dogs with fatal dyspnea (Erles et al., 2004; Decaro et al., 2010). Canine influenza virus (CIV) (Payungporn et al., 2008; Posuwan et al., 2010) and canine respiratory coronavirus (CRCoV) have recently been discovered from the respiratory tract of dogs with flu-like symptoms during a massive human flu outbreak (Erles et al., 2003; Erles and Brownlie, 2005; Decaro et al., 2007).

Diagnosis of CIRDC-associated virus(es) is important for giving the appropriate treatment plan, prognosis and preventive strategies. Various diagnostic tests are available for these infections. However, many are not practical due to their time-consuming process, poor specificity or sensitivity, and costly diagnostic tools (Jeoung et al., 2013). Thus, a rapid molecular technique is an appropriate method of choice for

CIRDC virus detection. Because multiple viruses cause CIRDC, including co-infections, a multiplex polymerase chain reaction (PCR) was developed and has become commercially available as a test for respiratory tract infections (Bellau-Pujol et al., 2005; Payungporn et al., 2006; Suwannakarn et al., 2008; Jeoung et al., 2013). Recently, multiplex real-time PCR (qPCR) has largely replaced the conventional counterpart in order to increase the sensitivity. However, it is challenging, in terms of financial support, in developing countries and so is limited in clinical and practical uses. Thus, using a multiplex PCR would be simple, sensitive and cost-effective to screen for CIRDC viruses. Accordingly, two multiplex PCR assays were developed in this study for the simultaneous detection of CIV, CPIV, CDV, CRCoV, CAdV-2, and CaHV-1.

MATERIALS AND METHODS

Positive control preparations

The positive control for CDV, CPIV and CAdV-2 was obtained from the modified-live vaccine Vanguard® plus 5/CV-L (Zoetis, Michigan, USA), containing CPIV ($10^{5.0}$ $TCID_{50}$/ m*l*), CDV ($10^{2.5}$ $TCID_{50}$/ m*l*) and CAdV-2 ($10^{2.9}$ $TCID_{50}$/ m*l*). Meanwhile, positive controls for CRCoV, CaHV-1 and CIV were derived from naturally infected dogs that were confirmed by nucleic acid sequencing. The H3N2 CIV positive control was kindly provided by Prof. Alongkorn Amornsin, Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University.

Specimens

Nasal (NS) and oropharyngeal swabs (OS) were collected from 51 suspected CIRDC suffering dogs; they were brought to veterinary hospitals residing in metropolitan Bangkok, Thailand, during February–August 2014. Those dogs that showed respiratory problems, such as nasal discharge, cough and evidence of bronchopneumonia were included, whereas those that revealed secondary respiratory disease caused by cardiovascular and/or functional tracheal disease were excluded from the study. Vaccination status of sampled dogs was also recorded.

After taking the NS and OS using sterile rayon tipped applicators (Puritan®, Maine, USA), the swabs were immersed in 1% phosphate buffer saline (PBS) and kept at -80 °C until assayed. The study protocol was approved by Chulalongkorn University Animal Care and Use Committee (No. 1431005).

Viral nucleic acid extraction, quantification and reverse transcription

Viral nucleic acid from the positive controls and specimens were extracted using the Viral Nucleic Acid Extraction Kit II (GeneAid, Taipei, Taiwan) according to manufacturer's recommendation. Nucleic acid was quantified and qualified using Nanodrop® Lite (Thermo Fisher Scientific Inc., Massachusetts, USA) at an absorbance of 260 and 280 nm to derive the A_{260}/A_{280} ratio. The extracted nucleic acid was divided into two aliquots, one for reverse transcription (RT) for detection of the RNA viruses (CIV, CPIV, CDV and CRCoV) and the other for a direct PCR assay for detection of the DNA viruses (CAdV-2 and CaHV-1). The RT was performed using 100 *n*g RNA as the template for complementary DNA (cDNA) synthesis using the Omniscript® Reverse Transcription Kit (*Qiagen* GmbH, Hilden, *Germany*). The cDNA and DNA were stored at -20 ºC until used for further PCR amplification.

Specific primers for viruses causing CIRDC

The sequences of the primers used for CAdV-2 (E3 gene), CDV (NP gene), CIV (M gene), CPIV (NP gene), CRCoV (S gene) and CaHV-1 (GB gene) amplification were retrieved from previous studies (Erles and Brownlie, 2005; Payungporn et al., 2008; Decaro et al., 2010; Posuwan et al., 2010) and are shown in Table 1. In order to ascertain the sensitivity, specificity and interaction of those primers, more than 45 sequences of each target gene were compared by multiple alignments using BioEdit Sequence Alignment Editor Version 7.1.3.0 (Ibis Biosciences, Carlsbad, CA, USA). The *in silico* specificity test was performed to select the conserved regions using BLASTn analysis in order to ensure the primer specificity without cross amplification of canine genes. Degenerate primers for CIV, CDV, CAdV-2 and CRCoV were applied (Table 1). Moreover, the canine glyceraldehyde-3-phosphate dehydrogenase

(GAPDH) gene was used as internal control as reported previously (Banoo et al., 2010).

Optimization of the simplex PCR

Prior to performing the PCR for detection of RNA viruses, a first round PCR for CRCoV was performed in order to increase the detection sensitivity. Reactions were comprised of a mixture of 2x GoTaq® Hot Start Green Master Mix (Promega, Wisconsin, USA), 0.4 μM final concentration of each outer primer (CoV_16053_F and CoV_16594_R) and 2 μl of cDNA, and made up to 25 μl with nuclease-free water. Reactions were performed using 3Prime G Gradient Thermal Cycle (Techne, UK). Cycling conditions were comprised of an initial denaturation at 94 ºC for 5 min, followed by 40 cycles of 95 ºC for 30 sec, 55 ºC for 30 sec and 72 ºC for 30 sec. The final extension was performed at 72 ºC for 7 min. Subsequently, the amplified CRCoV product of the first round PCR, and cDNA of the other RNA viruses (CIV, CPIV and CDV) and extracted DNA viruses (CAdV-2 and CaHV-1) were used as a template for further simplex PCR studies.

Gradient simplex PCR was performed for each virus. All reaction compositions were as mentioned above, but the gradient annealing temperature (Ta) was programmed ranging from 50 ºC to 59 ºC in order to optimize the reaction. Thermal cycling was performed with 95 ºC for 5 min, then 40 cycles of 95 ºC for 1 min, varied Ta for 1 min and 72 ºC for 1 min, and then finally 72 ºC for 10 min. The amplicons were resolved by 2% (w/v) agarose gel electrophoresis with 10% ethidium bromide in-gel staining and visualized by UV transillumination and compared to expected size of the PCR product (Table 1).

Table 1. Primers used for the PCR amplification of CIRDC viruses.

Virus	Primer name	Primer sequence (5' to 3')	Target gene[1]	Product size (bp)
CIV	CIV_M_F151	CATGGARTGGCTAAAGACAAGACC	M	126
	CIV_M_R276	AGGGCATTTTGGACAAAKCGTCTA		
CDV	CDV_N_F768	AACAGRRATTGCTGAGGACYTAT	NP	290
	CDV_N_R1057	TCCARRRATAACCATGTAYGGTGC		
CAdV-2	CadV_E3_F25073	TATTCCAGACTCTTACCAAGAGG	E3	551
	CadV_E3_R25623	ATAGACAAGGTAGTARTGYTCAG		
CPIV	CPIV_N_F428	GCCGTGGAGAGATCAATGCCTAT	NP	187
	CPIV_N_R614	GCGCAGTCATGCACTTGCAAGT		
CRCoV	CoV_16053_F	GGTTGGGAYTAYCCTAARTGTGA	S	542 (1^{ST} PCR)
	CoV_16594_R	TAYTATCARAAYAATGTCTTTATGTC		
	CoV_Pan_16510_R	TGATGATGGNGTTGTBTGYTATAA		458 (2^{ND} PCR)
CaHV-1	CaHV_GBF439	ACAGAGTTGATTGATAGAAGAGGTATG	GB	136
	CaHV_GBR574	CTGGTGTATTAAACTTTGAAGGCTTTA		

[1] M = Matrix, NP = Nucleoprotein, E3 = Early transcribed region, S = Spike protein, GB = Glycoprotein

Optimizations of multiplex PCR

The multiplex PCR was optimized separately for RNA- and DNA-associated CIRDC viruses. The starting genetic material for RNA virus detection was derived from two compartments: (1) product from the first nested PCR of CRCoV and (2) cDNA of the other RNA viruses. Reaction composition and condition were optimized as mentioned above for the simplex PCR. The suitable Ta for all RNA and DNA viruses were selected for further comparative analysis with simplex PCR.

Sequencing of PCR amplicons was performed to confirm their correct identity and thus the specificity of the PCR reaction. Amplicons were purified with a NucleoSpin Extract II (Macherey-Nagel, Düren, Germany) kit and submitted to The 1st BASE, Pte. Ltd. (Singapore) for direct sequencing. The derived nucleotide sequences were aligned using the BioEdit Sequence Alignment Editor version 7.0.9.0 software, and the respective consensus sequences were compared to those in the GenBank database using BLASTn analysis.

Analysis of specificity, sensitivity and reproducibility

Specificity test

The analytical specificity of each simplex PCR assay was evaluated by cross-reaction tests with various CIRDC-associated viruses, as well as canine parvovirus (CPV), canine enteric coronavirus (CCoV) and *Bordetella bronchiseptica.*

Sensitivity test

To access the analytical sensitivity of each simplex PCR assay, two-fold serial dilutions of nucleic extracted positive controls were amplified. The ten dilutions of tested controls were 2^{0}–2^{-10}*n*g/ PCR reaction.

Reproducibility

Both intra- and inter-assay variations were measured using the positive controls and sequenced clinical samples. To assess the intra-assay variation, triplicate amplifications of the 2^{-10} and 2^{0}*n*g/reaction templates for the positive controls and the samples were performed in a single multiplex PCR assay. To evaluate the inter-assay

variation, the above single multiplex PCR was performed as three independent multiplex PCR assays.

Diagnostic performance of the multiplex PCR

To evaluate the reliability of the developed multiplex PCR for clinical testing, the performance of the assay was compared to those of the simplex PCR and a commercial test kit (Antigen Rapid CIRD-3 Ag test kit, Bionote, South Korea). The sensitivity, specificity, positive predictive value and negative predictive value were determined. Independent t-test was used to evaluate the difference between route of sample collection and number of viral detection using SPSS 22.0 (IBM Corp, New York, USA)

RESULTS

Study population

The 51 dogs with respiratory clinical illness included in this study were 29 males and 22 females. Most of the dogs were puppies (37.3%) or senile (23.5%). Most presented with a nasal discharge (80.4%), coughing (47.1%), loss of appetite (56.9%) and bronchopneumonia (41.2%). Only 29.4% (15/51) of dogs were vaccinated.

Optimized and analytical performances of simplex and multiplex PCR assays

Optimization of each simplex PCR was undertaken using positive controls and clinical samples with different cycling conditions. Different annealing temperatures were evaluated, with the optimum Ta for all virus detections being 58 °C, at which temperature no primer dimers or non-specific amplicons were detected (data not shown). *In silico* and *in vitro* analytical specificity tests revealed that each primer was able to amplify the specific target DNA without any cross amplification among the CIRDC viruses, CPV, CCoV and *B. bronchiseptica*. In addition, the sequenced amplicons showed 100% sequence identity with their respective corresponding sequence in the GenBank database.

Analytical sensitivity, specificity and reproducibility

The sensitivity of the multiplex PCR was tested by detection of the various viruses in serial dilutions and compared with that using the simplex PCR for each particular virus. The multiplex PCR products of the tested viruses were observed at the same template dilutions as with the simplex PCR, suggesting a similar sensitivity for the simplex and multiplex PCRs (Figs. 1 & 2). The highest detection threshold was found for CDV and CRCoV, then CaHV-1 and CIV, and finally by CPIV and CAdV-2.

The specificity of the tested PCRs was evaluated by using other pathogens as mentioned above. No specific amplicons were detected in all reactions. For evaluation of the reproducibility, both intra- and inter-assay variations revealed similar results among the assays (data not shown).

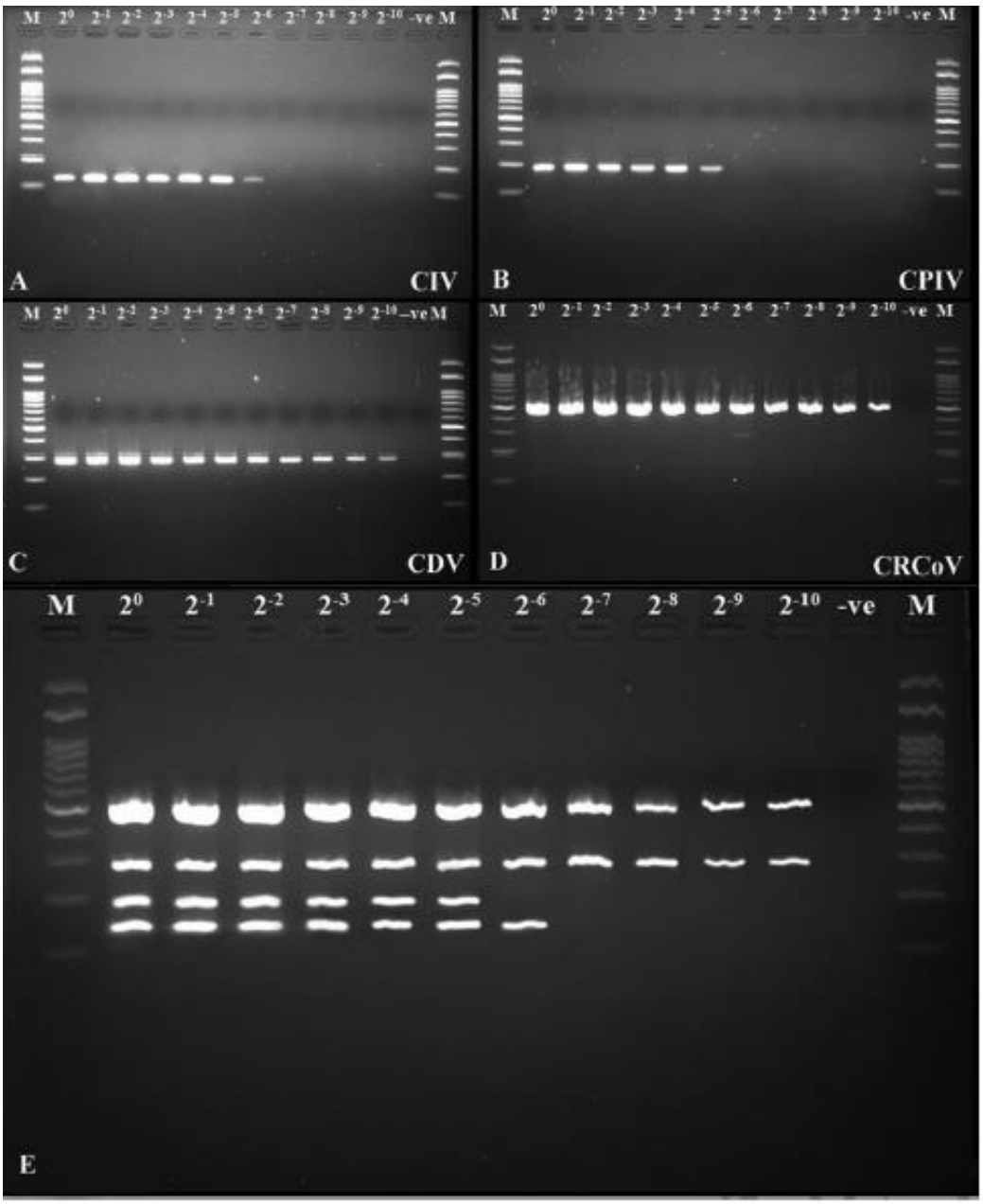

Figure 1. Analytical sensitivity test of the (A–D) simplex and (E) multiplex RT-PCR of RNA-associated CIRDC viruses. (A) CIV, (B) CPIV, (C) CDV and (D) CRCoV. Two-fold serial dilutions of the positive controls ranging from 2^{0} –2^{-10} ng/reaction were assayed. Detection threshold was equal in both the simplex and multiplex modalities and revealed minimal detectable dilution at 2^{-6} (CIV), 2^{-5} (CPIV) and ≥2^{-10} (CDV and CRCoV) ng/reaction. M= DNA marker 100 bp, –ve= negative control.

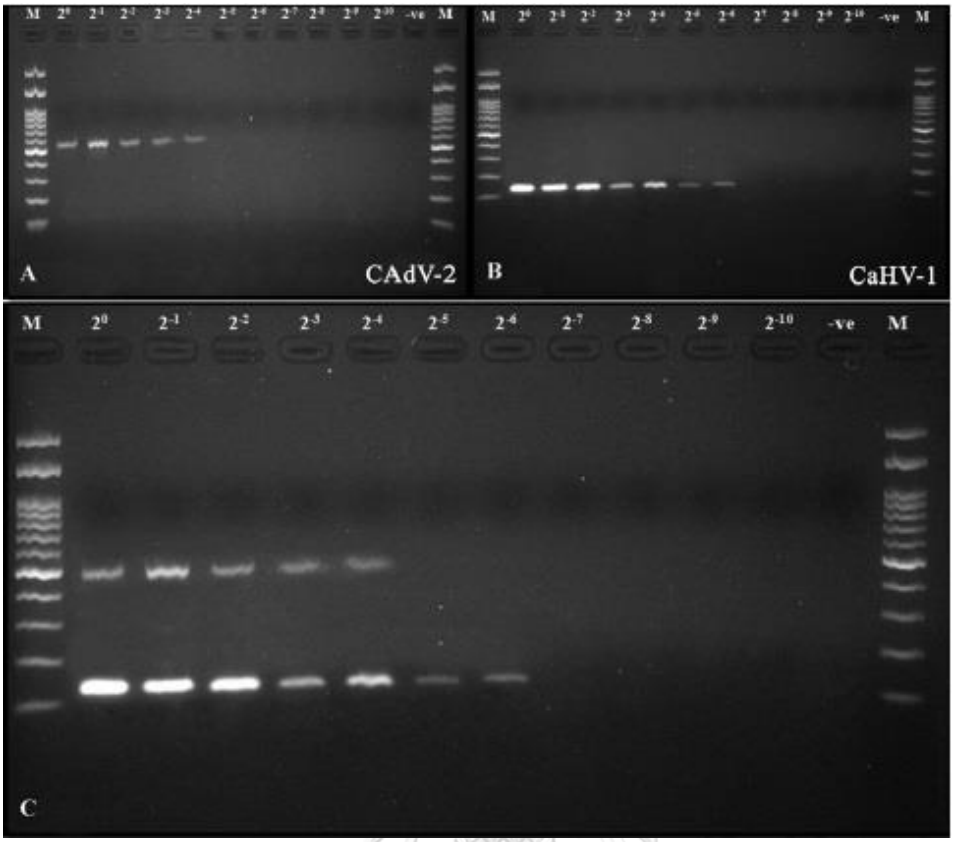

Figure 2. Analytical sensitivity test of (A, B) simplex and (C) multiplex PCR of DNA-associated CIRDC viruses. (A) CAdV-2 and (B) CaHV-1. Two-fold serial dilutions from 2^{0} -2^{-10} ng/reaction were tested. Detection threshold was similar in both the simplex and multiplex modalities and revealed minimal detectable dilution at 2^{-4} (CAdV-2) and 2^{-6} (CaHV-1) ng/reaction. M= DNA marker 100 bp, –ve= negative control.

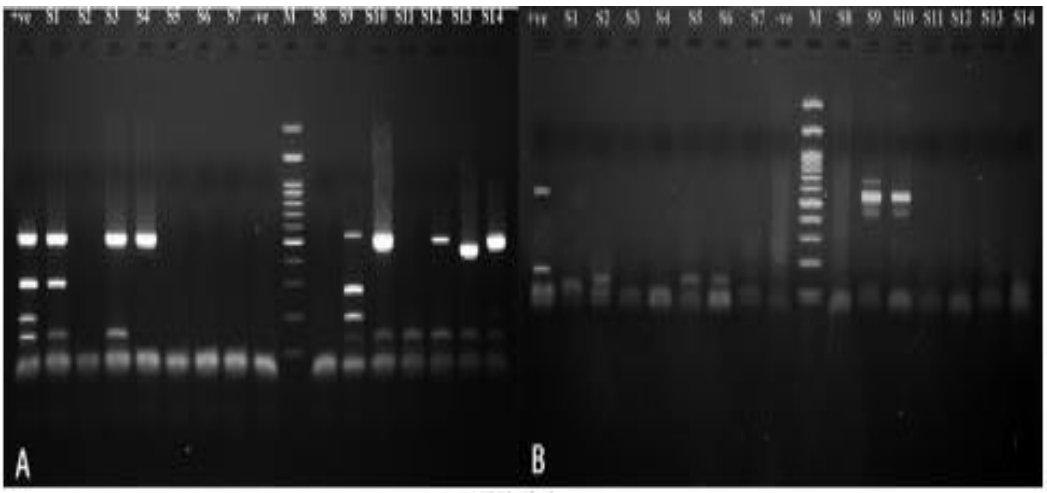

Figure 3. Results of the (A) multiplex RT-PCR and (B) multiplex PCR tested on clinical samples (S1–S14). M=DNA marker 100 bp, –ve= negative control, +ve= positive control.

Evaluation of the multiplex PCR using clinical specimens

The multiplex PCRs were tested on the 51 NS and 51 OS samples (Fig. 3) and compared with the simplex PCR assays for each respective virus (Table 2.). The CAdV-2 and CRCoV detection had 100% sensitivity and specificity for both the NS and OS sampling sites. False negative results were observed in CaHV-1, CIV, CPIV and CDV detection when performing multiplex PCRs, which resulted in a lower sensitivity of 87.5–97.7%. The PPV (100%) of all multiplex PCRs was consistent with the specificity (100%), while the NPV (89.5–99.0%) of those reactions was contrary with their sensitivity. Neither the multiplex RT-PCR nor the multiplex PCR showed false positive results when compared with its simplex counterpart.

The comparison between the multiplex PCRs and the rapid three-antigen test kit (CAdV-2, CIV and CDV) was performed on the same samples (Table 3.). With the clinical samples tested in this study, the rapid test kit yielded 100% sensitivity and a relatively high specificity for CAdV-2 and CDV. However, for CIV, there were high numbers of PCR-positive samples detected by multiplex PCR (83/102) whereas the test kits showed negative results.

Detection of CIRDC viruses in clinical samples by multiplex PCR

In single infections, CIV was the predominant virus detected and accounted for 23.5% (12/51) and 19.6% (10/51) positive NS and OS samples, respectively. The next most common virus was CPIV, detected at 3.9% (2/51) and 5.8% (3/51) of NS and OS samples, respectively, with 2% (1/51) being positive for CRCoV infection in both NS and OS samples. Even though the CDV, CAdV-2 and CaHV-1 were not detected as a single infection, they were detected in multiple infections in these tested samples (Table 4.).

For dual infections, the most frequently detected viruses were CIV co-infected with CRCoV at 13.7% and 21.6% in NS and OS, respectively, followed by CIV with CPIV at 9.8% and 7.8% in NS and OS samples, respectively. For triple infections, CIV and CRCoV were frequently found together co-infected with other viruses, and especially with CDV and CPIV. However, one dog was negative for all tested viruses in both the NS and OS sample.

Generally, dual infections were predominant in CIRDC suffering dogs (42.2%), followed by single (28.4%) and triple (22.6%) infections. With regards to the sampling site, the frequency of positive results was not statistically different between the OS and NS sampling sites ($P>0.05$).

Table 2. Comparison of the results from the simplex PCR and multiplex PCR for detection of CIRDC associated viruses in clinical samples.

		Simplex PCR				Total	Sensitivity	Specificity	PPV[3]	NPV[3]
CAdV-2		CAdV-2 pos[1]		CAdV-2 neg[1]						
		NS[2]	OS[2]	NS	OS					
Multiplex PCR	CAdV-2 pos	4	6	0	0	10				
	CAdV-2 neg	0	0	47	45	92				
	Total	10		92		102	100	100	100	100
CaHV-1		CaHV-1 pos		CaHV-1 neg						
		NS	OS	NS	OS					
Multiplex PCR	CaHV-1 pos	3	4	0	0	7				
	CaHV-1 neg	1	0	47	47	95				
	Total	8		94		102	87.5	100	100	99
CIV		CIV pos		CIV neg						
		NS	OS	NS	OS					
Multiplex RT-PCR	CIV pos	41	42	0	0	83				
	CIV neg	1	1	9	8	19				
	Total	85		17		102	97.7	100	100	89.5

[1]pos = positive, neg = negative. [2] NS = nasal swab, OS = oropharyngeal swab. [3]PPV = positive predictive value, NPV = negative predictive value

Table 2. Comparison of the results from the simplex PCR and multiplex PCR for detection of CIRDC associated viruses in clinical samples (continue).

		Simplex PCR				Total	Sensitivity	Specificity	PPV[3]	NPV[3]
CPIV		CPIV pos		CPIV neg						
		NS	OS	NS	OS					
Multiplex RT-PCR	CPIV pos	18	15	0	0	33				
	CPIV neg	1	2	32	34	69				
	Total	36		66		102	91.7	100	100	95.7
CDV		CDV pos		CDV neg						
		NS	OS	NS	OS					
Multiplex RT-PCR	CDV pos	14	13	0	0	27				
	CDV neg	2	1	35	37	75				
	Total	30		72		102	90	100	100	96
CRCoV		CRCoV pos		CRCoV neg						
		NS	OS	NS	OS					
Multiplex RT-PCR	CRCoV pos	23	23	0	0	46				
	CRCoV neg	0	0	28	28	56				
	Total	46		56		102	100	100	100	100

[1]pos = positive, neg = negative. [2] NS = nasal swab, OS = oropharyngeal swab. [3]PPV = positive predictive value, NPV = negative predictive value.

Table 3. Comparison of the results from the multiplex PCR and the rapid antigen test kit for the detection of CAdV-2, CIV and CDV in clinical samples.

		Rapid antigen test kit				Total	Sensitivity	Specificity	PPV[3]	NPV[3]
CAdV-2		CAdV-2 pos[1]		CAdV-2 neg[1]						
		NS[2]	OS[2]	NS	OS					
Multiplex PCR	CAdV-2 pos	0	1	4	5	10				
	CAdV-2 neg	0	0	47	45	92				
	Total	1		101		102	100	91.09	10	100
CIV		CIV pos		CIV neg						
		NS	OS	NS	OS					
Multiplex RT-PCR	CIV pos	0	0	41	42	83				
	CIV neg	0	0	10	9	19				
	Total	0		102		102	UC[4]	18.63	0	100
CDV		CDV pos		CDV neg						
		NS	OS	NS	OS					
Multiplex RT-PCR	CDV pos	6	6	8	7	27				
	CDV neg	0	0	37	38	75				
	Total	12		90		102	100	83.33	44.44	100

[1] pos = positive, neg = negative. [2] NS = nasal swab, OS = oropharyngeal swab. [3] PPV = positive predictive value, NPV = negative predictive value. [4] UC = unable to calculate.

Table 4. CIRDC viruses detected by multiplex PCR in the 102 clinical samples from 51 dogs.

Single infection (n = 29)	**NS[1] (n = 15)**	**OS[1] (n = 14)**
CIV	12	10
CPIV	2	3
CRCoV	1	1
Dual infection (n = 43)	**NS (n = 20)**	**OS (n = 23)**
CIV + CPIV	5	4
CIV + CDV	1	1
CIV + CRCoV	7	11
CIV + CAdV-2	2	1
CIV + CaHV-1	1	3
CPIV + CRCoV	1	0
CDV + CRCoV	3	2
CDV + CAdV-2	0	1
Triple infection (n = 23)	**NS (n = 13)**	**OS (n = 10)**
CIV + CPIV + CDV	2	2
CIV + CPIV + CRCoV	4	1
CIV + CPIV + CAdV-2	0	1
CIV + CDV+ CRCoV	3	3
CIV + CRCoV + CAdV-2	0	1
CIV + CRCoV + CaHV-1	1	1
CPIV + CDV + CRCoV	1	0
CPIV + CDV + CAdV-2	2	1
4 co-infection (n = 3)	**NS (n = 1)**	**OS (n = 2)**
CIV + CPIV + CDV + CRCoV	1	2
5 co-infection (n = 2)	**NS (n = 1)**	**OS (n = 1)**
CIV + CPIV + CDV + CRCoV + CAdV-2	0	1
CIV + CPIV + CDV + CRCoV + CaHV-1	1	0
Negative (n = 2)	**NS (n = 1)**	**OS (n= 1)**
	1	1
Total	*51*	*51*

DICUSSION

The CIRDC is an important disease that impacts on dogs, especially puppies or immunosuppressed dogs, and is frequently associated with viral infections. It has gained attention recently because many viruses have been discovered and co-infections with multiple pathogens is often fatal. Thus, the development of diagnostic tools for CIRDC-associated virus detection is necessary to enhance the diagnosis coverage. In this study, multiplex RT-PCR and multiplex PCR for the detection of CIRDC-associated RNA and DNA viruses, respectively, were developed and compared with conventional methods. Both developed multiplex PCRs could detect several viruses associated with CIRDC efficiently. The two multiplex PCRs gave similar results equivalent to that obtained from the conventional simplex PCRs that could only detect one pathogen per reaction and so required six separate reactions per sample. Nested amplification was performed for CRCoV detection in order to increase the sensitivity of detection (Poovorawan, personal communication). Although multiplex PCR has been developed previously to detect several pathogens of CIRDC, such as CIV, CDV and CRCoV (Jeoung et al., 2013), its application remained limited because of the narrow range of viruses covered, with other CIRDC-associated viruses being neither detected nor ruled out. Thus, our study might provide a novel platform for whole CIRDC-virus detection.

The overall sensitivity of the multiplex RT-PCR and multiplex PCR was more than 90% and 87%, respectively, compared to their simplex counterparts. However, the detection of CRCoV was modified as a hemi-nested RT-PCR to increase its sensitivity. The false negative reactions when performing multiplex PCRs in this study might be resulted from the selection of the single optimized Ta for several primer pairs and the low amount of particular target genes (Bellau-Pujol et al., 2005). These suggested for the decreased sensitivity of the developed multiplex PCRs. Moreover, there was 100% specificity in both modalities for clinical sample detection. Thus, these platforms could likely be used effectively in practice. Recently, some multiplex PCR assays were developed in order to detect the CIRDC pathogens (Jeoung et al., 2013); however, the test might be immature because only CIV, CDV, and CRCoV could be

detected but not for others. Thus, our study expanded the coverage of CIRDC virus detection. In an evaluation of the commercially available three-antigen rapid test kit (CAdV-2, CIV and CDV), we found only CIV detection showed an unexpected sensitivity and specificity. A previous study reported that the developed multiplex RT-PCR for H3N2 CIV, CDV and CRCoV detection had an almost 100% sensitivity and specificity compared with the conventional RT-PCR and rapid antigen test kit (Jeoung et al., 2013). In contrast, our study showed that the CIV-positive samples by multiplex RT-PCR were negative when tested with the rapid antigen test kit. This is consistent with reports that many rapid test kits might have a low sensitivity to detect the influenza virus, but could still be suitable for rapid in-house clinical applications (Liao et al., 2011; Pecoraro et al., 2013). This reflects that the type of kit, viral copy number, duration of storage, route of sample collection, and type or virus strain may all influence the test results (Bellau-Pujol et al., 2005). Interestingly, in this study about 70% (71/102) of samples from the clinical respiratory illness dogs were found to have multiple infections. This finding supports that symptomatically the CIRDC is a complex disease, which is mostly caused by co-infection with more than one pathogen. Recently, Jeoung et al. (2013) used both NS and whole blood samples for CIRDC virus detection, but found that only CDV (and not CIV and CRCoV) could be detected from the whole blood samples (Jeoung et al., 2013). Correspondingly, respiratory swabs have been reported to be appropriate samples for the detection of respiratory pathogens (Posuwan et al., 2010; Gritzfeld et al., 2011). Thus, NS and OS served as appropriate sample sources in our study due to their ease of and non-invasive sampling nature and that they lie on the viral shedding routes. This study also suggested that the virus should be screened for in NS and OS, with detection levels at each site depending on the type of virus. The CAdV-2 and CaHV-1 mostly replicate in the lower respiratory tracts and shed via respiratory discharge, consisting with our finding that they were mostly detected in the OS even though NS could often detect these viruses as well. However, the CAdV-2 primer pair used in this study was able to amplify CAdV-1 DNA virus which also shows airborne transmission and replicates in tonsil (Buonavoglia and Martella, 2007). Therefore, the positive PCR reaction for canine adenovirus could not discriminate between CAdV-1 and CAdV-2 in this study. Additionally, CaHV-1 can be latent in various

nerve ganglions, resulting in negative results from nucleic acid-based CaHV-1 detection in respiratory discharges in non-symptomatic dogs (Miyoshi et al., 1999).

In this study, 3 out of 15 vaccinated dogs receiving, at least once, combined vaccine against CPIV, CDV and CAdV-2 showed PCR positive results for CIRDC virus detection (2 CDV positive dogs and 1 CPIV positive dog). Even though live attenuated vaccines can give false positive results with molecular testing, it is essential to discriminate between wild-type infection and recent vaccination for the prevention of false positivity. However, Radtanakatikanon et al (2013) has been shown the only natural CDV infection in CDV infected and vaccinated dogs by means of F-, H- and P-gene RT-PCRs and restriction fragment length polymorphism (RFLP) (Radtanakatikanon et al., 2013). Another one CPIV positive vaccinated dog is needed to be investigated in the future.

This study documented CaHV-1 and CRCoV circulation in Thailand for the first time. In 2012, CIV H3N2 was discovered in Thailand from dogs with flu-like symptoms (Bunpapong et al., 2014). Here, CIV and CRCoV were the most frequently detected viruses in CIRDC-infected dogs, suggesting that the viruses might spread rapidly. These viruses were not only found in single infections but they were also found as co-infections together or with other viruses.

This study also exhibited a higher level of infections compared with a previous report (Pecoraro et al., 2013), although this might be caused by the different timing of sample collection, population size and locations. However, it has previously been reported that infection with CRCoV and CPIV might facilitate or initiate the disease and, subsequently, enhance the entry of other pathogens (Erles et al., 2004), and so the prevalence of infected dogs is then increased. Moreover, we found that the dogs that were infected with CIV, CPIV, CDV, CRCoV showed a greater severity of clinical symptoms, such as marked bronchopneumonia and sudden death (data not shown). This finding is consistent with other investigations suggesting that co-infections might augment the severity of clinical symptoms (Erles et al., 2004; Posuwan et al., 2010). Thus, advanced genetic-based detection methods, such as multiplex PCR assays, are considered as an alternative diagnostic platform for a panel of suspected CIRDC causing viruses with a high sensitivity and specificity. Because of the cost benefit and practical

usage, the developed multiplex PCR assays are suitable for a screening test for disease diagnosis, quarantine and prevention measures, especially in developing countries.

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2.2 VIRAL MOLECULAR AND PATHOLOGICAL INVESTIGATIONS OF *CANID HERPESVIRUS* 1 INFECTION ASSOCIATED RESPIRATORY DISEASE AND ACUTE DEATH IN DOGS

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ABSTRACT

Canid herpesvirus 1 (CaHV-1) is a member of the canine infectious respiratory disease complex (CIRDC). The outcome of CaHV-1 infection can be occasionally fatal. So far, no information on CaHV-1 circulation in Thailand has been reported resulting in a lack of preventive strategies. In this study, nasal (NS) and oropharyngeal (OS) swabs were collected from 100 live dogs with respiratory distress. Among them, 23 pleural effusions were aspirated. A panel of CIRDC-associated viruses was screened by (RT)-PCR, including CaHV-1, CIV, CPIV, CDV, CRCoV and CAdV-2, for all collected samples. The CaHV-1 was detected in 32 dogs. Additionally, CaHV-1 was consistently detected in six pleural effusions. Most CaHV-1 infected dogs were over 5 years of age (43.8%) and expressed mild nasal discharge. Pathological results of four three-month-old puppies, naturally moribund from respiratory disease, revealed severe multifocal necrotic-hemorrhagic disease in several organs without pathognomonic inclusion bodies. They were only found to be CaHV-1 positive by PCR. Phylogenetic analysis demonstrated concordant results of CaHV-1 circulation in Thailand. Although mostly found as a co-infection with other CIRDC viruses (68.8%) also occurred alone. Therefore, rapid ante-mortem diagnosis might facilitate the investigation of unclassical CaHV-1 infection, which is fatal in neonates and causes illness in annually core-vaccinated adults.

KEYWORDS: *Canid herpesvirus* 1, CIRDC, pathology, respiratory distress, Thailand

INTRODUCTION

Canid herpesvirus 1 (CaHV-1) is an enveloped double stranded DNA virus that belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus* (Gaskell and Willoughby, 1999). The CaHV-1 has been defined as a member of the canine infectious respiratory disease complex (CIRDC), causing respiratory distress, such as nasal discharge, coughing and sometimes pneumonia, in infected dogs. Several studies have revealed that the co-infection of CaHV-1 with other CIRDC viruses, such as canine influenza (CIV), canine parainfluenza virus (CPIV), canine distemper virus (CDV), canine respiratory coronavirus (CRCoV) and canine adenovirus type 2 (CAdV-2), often leads to an increased severity of the symptoms. However, the role of CaHV-1in CIRDC remains unclear (Binn et al., 1979; Erles et al., 2004).

Infection of dogs by CaHV-1 may be transplacental or after direct contact with infected samples, such as uterine secretions during birth or oro-nasal secretions. Infection during the mid- to late-gestation period might cause abortion or a mummified fetus. The incubation period of CaHV-1 varies from 6–10 days. The virus can evade the host immune response and persist in various nerve plexuses, resulting in latent infections and virus shedding when the host is immunocompromised (Okuda et al., 1993; Miyoshi et al., 1999). Clinical manifestations are dependent on the age and immune status of the host, where infected adult dogs are typically asymptomatic or show mild rhinitis and pharyngitis, while infected neonates or young puppies might die within a few days after birth or infection (Hashimoto et al., 1982; Carmichael et al., 1995).

After necropsy, the distinct lesion of CaHV-1 is seen as a generalized necrotizing and hemorrhagic disease, most obviously in the vital organs, such as the lung, liver, kidneys and spleen(Gadsden et al., 2012; Kumar et al., 2014), although a similar finding is also evident in other organs, including the small intestine, adrenal glands, brain, heart, pancreas, stomach and eyes. Eosinophilic intranuclear inclusion bodies might be present in the epithelial cells of the trachea, bronchi, lung, liver and kidneys, suggesting that inclusion bodies might be consistent with a fulminant CaHV-1 infection based on a high infection level of viral genomic DNA (Larsen et al., 2015).

Taxonomically, CaHV-1 appears to be a monotypic virus that is genetically similar to feline herpesvirus-1 (FHV-1), phocid herpesvirus-1 (PhHV-1) and equine herpesvirus-1 (EHV-1) and -4 (EHV-4) (Rota and Maes, 1990; Remond et al., 1996). However, the host range of CaHV is restricted to dogs only. This virus multiplies only in permissive canine-derived cell lines, such as Madin-Darby canine kidney (MDCK) cells. Two stages in CaHV-1 attachment and invasion *in vitro* have been suggested. Firstly, the virus interacts with heparan sulfate on the cell surface, and it is then secondly internalized via an unidentified viral component and cellular receptor. In contrast, the virus appears to attach to non-permissive cells through the heparin-sensitive mechanism but does not enter the cell due to the lack of the correct cellular receptor (Nakamichi et al., 2000).

Although there have been some reports to suggest that the respiratory problems in CaHV-1-infected dogs can be self-limited or even fatal worldwide (Kawakami et al., 2010), the fundamental data regarding CaHV-1 circulation in Thailand remains unknown. This study aimed to determine the prevalence of the CaHV-1 infections with genetic characterization in live respiratory distressed dogs as well as CaHV-1 investigations in acute dead puppies.

MATERIAL AND METHODS

Study population and sample collection

Live animals: one hundred dogs showing respiratory distress that had been brought to veterinary hospitals/clinics located in Bangkok, Thailand during 2013–2014 were included in this study. Nasal (NS) and oropharyngeal swabs (OS) were collected by sterile rayon tipped applicators (Puritan®, Maine, USA) and kept in sterile phosphate saline buffer. Among them, 23 pleural effusions (PE) diagnosed by radiographs were aspirated and collected in sterile 1.5-ml eppendorf tubes. All specimens were kept at-80°C until used in the molecular assay. Clinical signs were scored by certified veterinarians at the time of sampling as: +, nasal discharge; ++ mild cough; +++, cough and nasal discharge; ++++, cough, nasal discharge and inappetence; +++++, evidence of bronchopneumonia. Signalment, such as age and

history of vaccination, were recorded for further analysis. All following procedures were approved by the Chulalongkorn University Animal Care and Use Committee (No. 1431005).

Additionally, two bitches that delivered dead puppies were sampled by NS, OS and vagina swabs (VS) at the first week and 3 months after parturition. All swabs were then screened for CaHV-1 by genomic DNA detection using polymerase chain reaction (PCR).

Pathological study

Dead animals: four naturally moribund puppies, aged up to 3 months, which had suffered from severe sudden respiratory distress followed by epistaxis, were submitted for routine necropsy at the Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University. Clinical data and gross findings were recorded. Swabbed samples, including NS, OS and PE, were collected for molecular assay. Various tissues, including the brain, lung, trachea, bronchial lymph node, tonsil, liver, spleen, kidneys and intestines, were collected in 10% neutral buffered formalin for histopathology. Tissues were subjected to routine histological process and embedded in paraffin wax. Sections were cut at a 4-μm thickness, stained with hematoxylin and eosin (H&E), and then observed under a light microscope.

Virus detection

All sampled swabs (NS, OS and VS) as well as PE were screened for CaHV-1 by PCR. Viral nucleic acids were extracted using a Viral Nucleic Acid Extraction Kit II (GeneAid, Taipei, Taiwan) according to the manufacturer's recommendations. Genomic DNA was quantified and qualified using a Nanodrop™ Lite Spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, USA.). Each PCR reaction was performed in a final volume of 25 µl and consisted of GoTaq® Hot Start Green Master Mix (Promega, Wisconsin, USA), DNA template, nuclease-free water and specific primers as previously described (Decaro et al., 2010). The PCR thermal cycling was performed at 95ºC for 5 min, followed by 40 cycles of 1 min each at 95ºC, 58ºC and 72ºC, and then a final 72ºC for 10 min. After resolution by electrophoresis through a 2% (w/v) agarose gel, the PCR products (136 bp) were stained with 10% ethidium bromide and visualized

under an ultraviolet illuminator. Potential co-infection with other CIRDC-related viruses (CIV, CPIV, CDV, CRCoV and CAdV-2) was ascertained using multiplex PCR (for DNA viruses) and multiplex reverse transcriptase PCR (RT-PCR; for RNA viruses) approaches (Table 5.) (Piewbang et al., 2016).

Briefly, the extracted nucleic acids were divided into two samples. One pair of the nucleic acids was subjected to cDNA synthesis using the Omniscript® Reverse Transcription Kit (*Qiagen* GmbH, Hilden*, Germany*) according to manufacturer's recommendations. The cDNA was then used as a template for CIRDC-RNA virus detection by multiplex RT-PCR. The reaction was comprised of a mixture of 2x GoTaq® Hot Start Green Master Mix (Promega, Wisconsin, USA), 0.4 μM final concentration of each primer (Supplement Table 1) and 2 μl of cDNA, and made up to 25 μl with nuclease-free water. The reactions were performed using 3Prime G Gradient Thermal Cycle (Techne, UK). PCR was performed at 95ºC for 5 min, followed by 40 cycles of 1 min each at 95ºC, 58ºC and 72ºC, and then a final 72ºC for 10 min. The other pair of nucleic acids was subjected to PCR assay for CAdV-2 detection with the same cycling conditions as described above in the multiplex RT-PCR. The PCR products were resolved by 2% (w/v) agarose gel electrophoresis with 10% ethidium bromide in-gel staining and visualized by UV transillumination and compared to expected size of the PCR product (Table 5). Likewise the potential infection with bacteria was screened from the tracheal discharge samples collected from the necropsied puppies.

Table 5. Primers for PCR amplification of CIRDC viruses (Piewbang et al., 2016).

Virus	Primer name	Primer sequence (5'-3')	Target gene*	Product size (bp)
Canine influenza virus (CIV)	CIV_M_F151	CATGGARTGGCTAAAGACAAGACC	M	126
	CIV_M_R276	AGGGCATTTTGGACAAAKCGTCTA		
Canine distemper virus (CDV)	CDV_N_F768	AACAGRRATTGCTGAGGACYTAT	NP	290
	CDV_N_R1057	TCCARRRATAACCATGTAYGGTGC		
Canine adenovirus type 2 (CAdV-2)	CAdV_E3_F25073	TATTCCAGACTCTTACCAAGAGG	E3	551
	CAdV_E3_R25613	ATAGACAAGGTAGTARTGYTCAG		
Canine parainfluenza virus (CPIV)	CPIV_N_F428	GCCGTGGAGAGATCAATGCCTAT	NP	187
	CPIV_N_R614	GCGCAGTCATGCACTTGCAAGT		
Canine respiratory coronavirus (CRCoV)	CoV_16053_F	GGTTGGGAYTAYCCTAARTGTGA	S	542 (First round PCR)
	CoV_16594_R	TAYTATCARAAYAATGTCTTTATGTC		
	CoV_Pan_16510_R	TGATGATGGNGTTGTBTGYTATAA		458 (Second round PCR)
Canine herpesvirus (CaHV)	CaHV_GBF439	ACAGAGTTGATTGATAGAAGAGGTATG	GB	136
	CaHV_GBR574	CTGGTGTATTAAACTTTGAAGGCTTTA		

* M = Matrix, NP = Nucleoprotein, E3 = Early transcribed region, S = Spike protein, GB =Glycoprotein

Phylogenetic analysis

Six selected CaHV-1 PCR products from necropsied dogs were purified using a NucleoSpin Extract II™ kit (Macherey-Nagel, Düren, Germany) and then commercially sequenced at Solgent Co., Ltd. (Korea) for genetic sequencing. The nucleotide sequences were aligned using the BioEdit Sequence Alignment Editor Version 7.0.9.0 software and then compared with previously deposited nucleotide sequences in the GenBank database using the BLASTn algorithm. Phylogenetic analysis was performed using the Maximum Likelihood (ML) algorithm using the BioEdit Sequence Alignment Editor Version 7.0.9.0 software. Standard errors were calculated by the Bootstrap method using 1,000 replicates (Radtanakatikanon et al., 2013).

RESULTS

Clinical relevance of CaHV-1 infection

In total, CaHV-1 was detected in 32 out of 100 live dogs with respiratory distress, which were comprised of 17 dogs (53.1%) that were positive for both OS and NS, while nine and six dogs were only positive from the NS (28.1%) and OS (18.8%), respectively. In addition, CaHV-1 was detected in 6/23 (28.3%) of the PE samples. Infection frequencies of live dogs with CaHV-1 showed an age-dependent affect, being highest in dogs of more than 5 years old (14/32, 43.8%), and decreasing with decreasing age at 8/32 (25%), 5/32 (15.6%) and 5/32 (15.6%) in dogs of 1–5 years old, 3 months to 1 year old and <3 months old, respectively. The clinical expressions of CaHV-1-positive dogs varied from mild to severe symptoms. They mostly expressed a nasal discharge (9/32, 28.1%), followed by nasal discharge with a cough (7/32, 21.9%), nasal discharge with a cough and inappetence (6/32, 18.8%), bronchopneumonia (6/32, 18.8%) and, to a lesser extent, a cough only (4/32, 12.5%). In relation to their vaccination history, for which commercial vaccines are only available for CPIV/CDV/CAdV-2 prevention, a higher frequency of CaHV-1 infection was found in vaccinated dogs (20/32, 62.5%) than in unvaccinated dogs (12/32, 37.5%).

Single CaHV-1 infection and multiple infections with other CIRDC-associated viruses

Single CaHV-1 infections were observed in 31.3% (10/32) of dogs, whereas co-infections with at least one other CIRDC virus were found in 68.8% (22/32) of dogs (Table 6., Fig. 4). Focusing only on the CaHV-1 positive dogs, the most frequent observations were found in adult dogs of >5 years old (5/10, 50%), minimally one-shot vaccinated (7/10, 70%), and clinically presenting with mild respiratory signs, such as nasal discharge (3/10, 30%). However, this pattern of findings was similar in the co-infected CIRDC dogs, since they were most frequent in dogs of >5 years old (8/22, 32.4%), vaccinated dogs (13/22, 59.1%) and those presenting with only a nasal discharge (6/22, 27.3%).

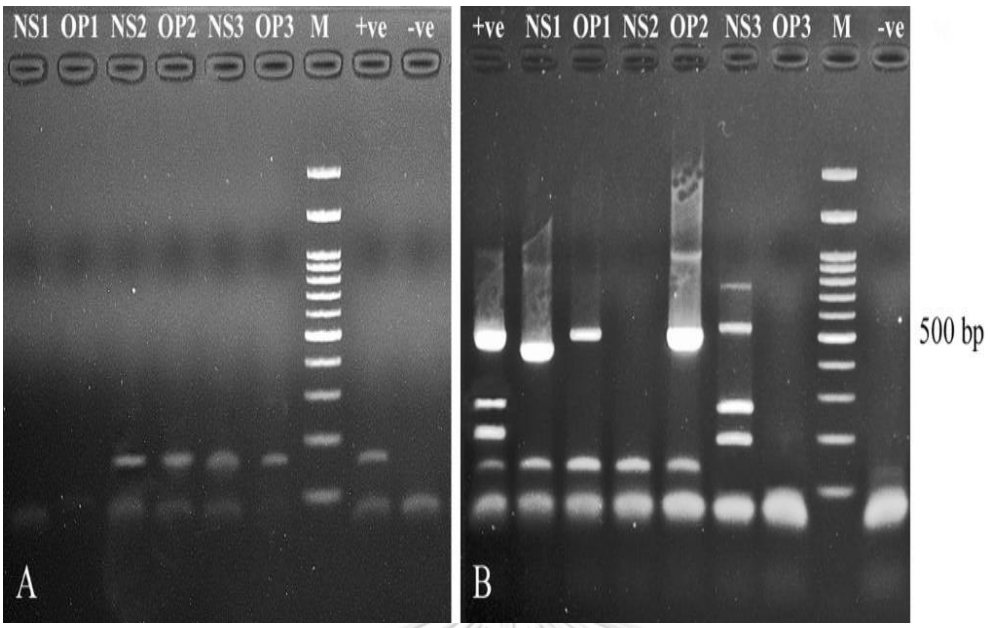

Figure 4. Canine infectious respiratory disease complex (CIRDC) associated viruses detected by (RT)-PCR. (A) Multiplex PCR showed the amplicon specific band of CaHV-1 (136 bp), but not CAdV-2 (551bp), in both nasal (NS) and oropharyngeal (OS) swabs of dog No. 2 and 3. (B) Multiplex RT-PCR revealed multiple infections of CIV (126 bp) and CRCoV (458bp) in dog No. 1 and 2, while CPIV (187 bp), CDV (290 bp) and CRCoV were found in the NS of dog No. 3. M: 100 bp DNA marker, +ve: positive control, -ve: negative control. Gels shown are representative of those seen from 2 independent PCR reactions.

Pathological findings of CaHV-1infected puppies

After necropsy, the macroscopic findings for the four puppies showed that more than 80% of the lungs were dark red, firm, edematous, non-collapsible and with blood oozing on the cut surface. Diffusely mottled white foci were infiltrated in the parenchyma (Fig. 5A). The tracheas were fully occupied with frothy, turbid fluid throughout the lumen and particularly in the distal part. All puppies also had 30–45 ml intrathoracic serosanguineous fluid. Reddish enlarged tracheobronchial lymph nodes and tonsils were presented. Their livers were enlarged, congested, firm and dark rubbery with diffusely small white foci (Fig. 5B). A diffusely patchy hemorrhage on the

kidney surface and multiple wedge-shaped hemorrhages at the renal medullar layer were evident on the cut surface (Fig. 5C). The spleen was enlarged with diffusely pinpoint reddish foci in all four puppies (Fig. 5D). Moderate congestion of the brain and intestines was noted.

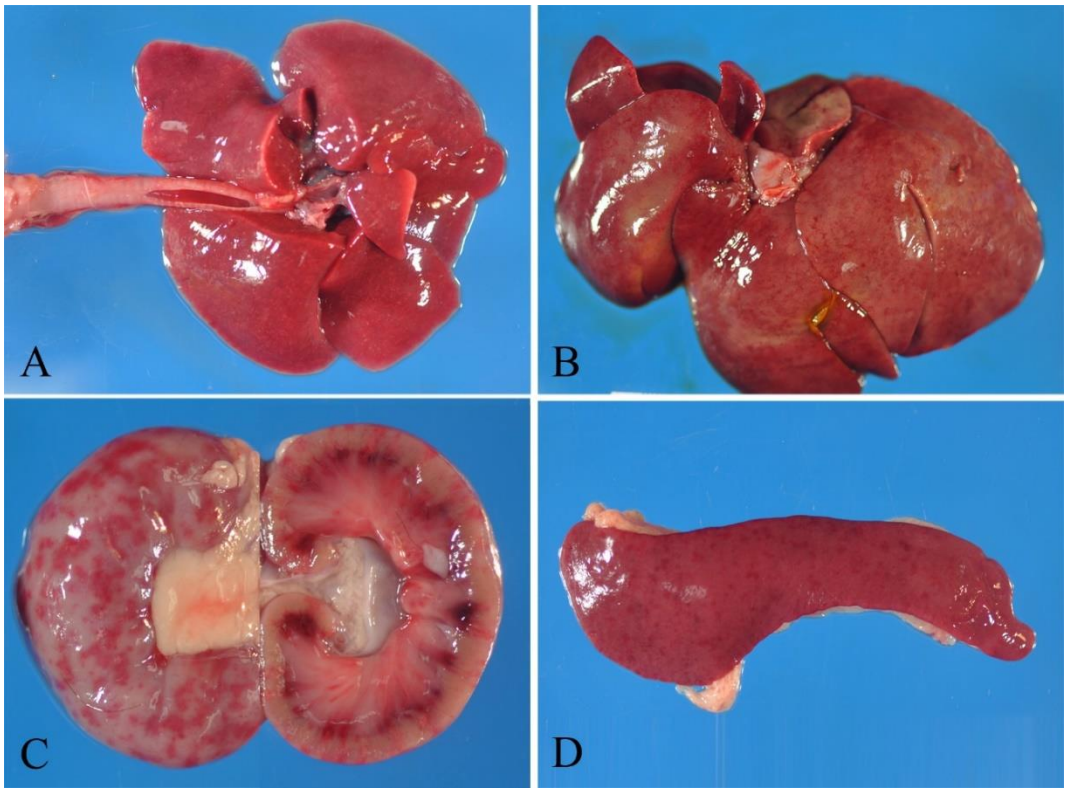

Figure 5. Post-mortem examination of CaHV-1 infected puppies. (A) a dark red enlarged lung with diffusely pinpoint white foci, (B) liver congestion with diffusely white foci, (C) diffuse patchy hemorrhage on the renal surface and multiple wedge-shaped hemorrhages at the renal medulla on its cut surface and (D) marked splenomegaly with diffusely pin point red foci.

Histologically, prominent lesions composed of severe acute multifocal necrotizing pneumonia with pulmonary congestion were evident in the lung tissue (Fig. 6A). Inflammatory cells, such as neutrophils, macrophages and some plasma cells, were infiltrated in the alveoli and pulmonary parenchyma. Moderate squamous

metaplasia of the bronchiolar epithelium was often observed and moderate fibrinosis was generally trapped in both the alveoli and lumen of the small bronchioles. Bronchioles were edematous with mild bronchial epithelial lacerations that resulted in sloughing of such cells and tissue debris into the airways. No evidence of obvious foreign organisms or inclusion bodies was noted. Moderate hemorrhagic histiocytic lymphadenitis of the tracheobronchial lymph nodes and tonsillitis were observed. For the liver, severe multifocal necrotizing hepatitis with hepatic congestion was markedly seen. In addition, two of four dogs also revealed moderately centrilobular fatty degeneration. In the kidney, severe acute tubular necrosis with massive congestion at both cortex and medulla were described (Fig. 6B). Multiple necrotic foci with histiocytic splenitis were noted. Mild nonsuppurative encephalitis was characterized by a few layers of mononuclear cell perivascular cuffing. Moderate nonsuppurative enteritis accompanied by a mild degree of villous atrophy was also seen.

Moreover, the NS, OS and PE samples from all necropsied puppies were found to be CaHV-1 positive by PCR detection. Co-infection with bacteria or other CIRDC viruses (CIV, CPIV, CDV, CRCoV and CAdV-2) was not detected in the respiratory samples by bacterial identification and PCR assays.

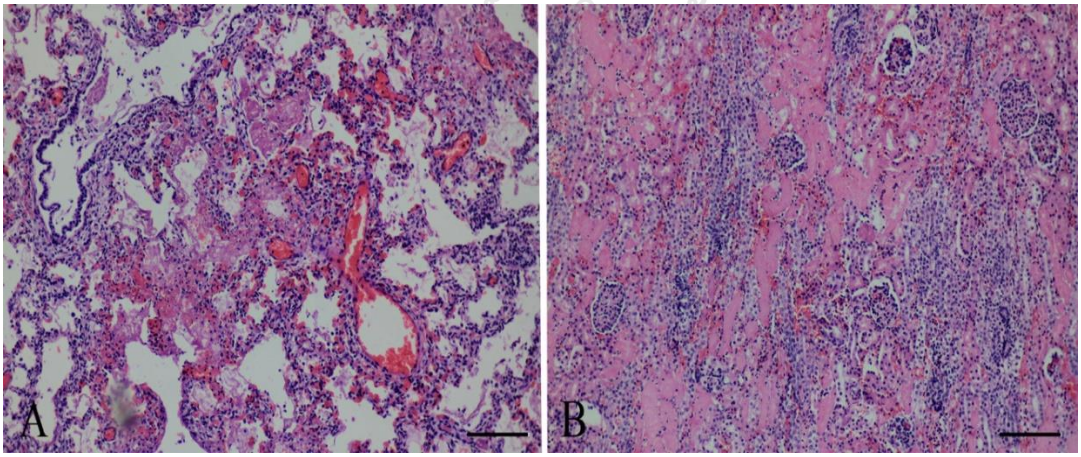

Figure 6. Histopathology of CaHV-1 infected puppies. (A) Severe acute multifocal pyogranulomatous necrotizing hemorrhagic pneumonia and (B) severe acute tubular necrosis with massive congestion. Bar = 200 μm.

Interestingly, we were informed later by the owners that two bitches of these four dead puppies had been sick during pregnancy. One bitch showed mild nasal discharge during the first 2 weeks of pregnancy and the other had diarrhea in the first 3 weeks of pregnancy. After CaHV-1 positive PCR results were reported for these puppies, the NS, OS and VS of the respective bitches were collected at the first-week and 3-months after parturition and screened for viruses. The presence of CaHV-1 DNA was detected in all the first-week samples, but was absent in all the 3-month specimens (data not shown).

Phylogenetic analysis

The sequences of the six selected amplified PCR products (CU1–6/TH/CaHV-1) displayed 100% homology to CaHV-1 strain KS-1 glycoprotein B (gB) gene (Accession number HQ846625), and were unrelated with other herpesviruses, suggesting that they are species specific. The phylogenetic analysis revealed the CaHV-1 circulating in Thailand (CU1–6/TH/CaHV-1) belonged to the *Alphaherpesviridae* and were genetically close to the PhHV-1 (Accession number Z68147) and Bovine herpesvirus 1 (Accession number JN787952) (Fig. 7).

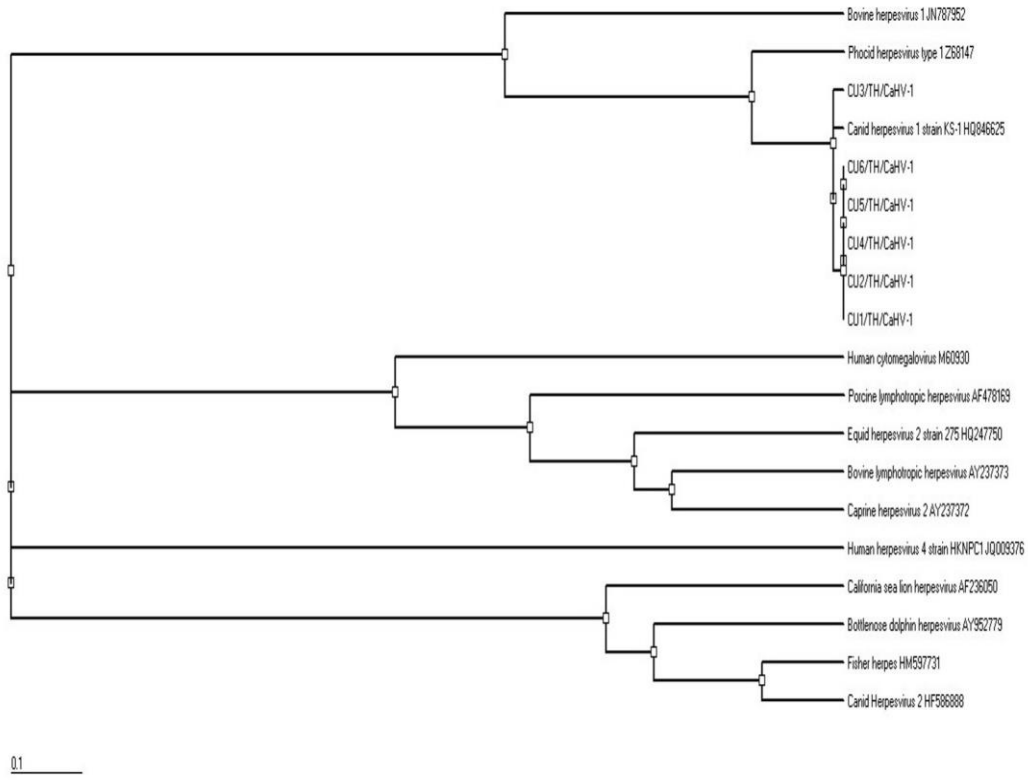

Figure 7. Maximum likelihood phylogenetic tree of CaHV-1 based on the 136 bp nucleotide sequence of the partial glycoprotein B gene. The CU1–6/TH/CaHV-1 isolates represent the six selected PCR products of CaHV-1 isolates in dogs in Bangkok, Thailand. They were compared with other herpesviruses from the indicated various hosts followed by their accession number.

DISCUSSION

Infection of dogs with CaHV-1 can manifest as a fatal systemic disease in neonatal dogs, particularly those of less than 6 weeks of age. Recently, CaHV-1 infection in clinically healthy adult dogs has been reported to cause severe acute respiratory problems (Kumar et al., 2014), and hepatic necrosis without any respiratory signs (Gadsden et al., 2012). However, the prevalence was quite rare. Despite that CaHV-1 has been recognized as one of the pathogens that causes CIRDC for decades, the prevalence of CaHV-1 infections circulating in Thailand has not been previously determined. This might be the result of a lack of CaHV-1 diagnostic tools in clinical use. In addition, this study also illustrated the fatal CaHV-1 infection associated with severe hemorrhagic and necrotizing respiratory disease in puppies. These findings showed evidence of unclassical CaHV-1 infection which was confirmed by the PCR analysis.

Viruses belonging to the *Herpesviridae* family are well-recognized pathogens that are able to potentially persist in their particular hosts. The CaHV-1 is extremely good at evading the host immune responses and is adapted for persistence in the peripheral ganglion of the nervous system at immune-privileged sites (Miyoshi et al., 1999). Later, the dormant virus reactivates for its replication and virion production without being recognized by host immune cells. The virus can spread its descendants from the infected organs in the lytic cycle when the host loses a proper immune function, such as under stress, pregnancy, anti-cancer drug use and systemic corticosteroid treatment (Malone et al., 2010; Radtanakatikanon et al., 2013). In addition, the latent infection of CaHV-1 can be reactivated during other infectious conditions, and particularly with CIRDC (Erles et al., 2004).This is in agreement with our findings showing that most of the CaHV-1 infected dogs were co-infected with other CIRDC-viruses. Multiple CIRDC virus infections often increase the severity of the clinical outcome (Erles et al., 2004). In addition, most routinely vaccinated dogs that were CaHV-1 positive and co-infected with CIV or CRCoV exhibited a low degree of severe respiratory problems, while those that were unvaccinated and simultaneously infected with CDV and CAdV-2 expressed progressive clinical signs (data not shown). These

findings might have resulted from the lack of neutralizing immunological responses against CDV and CAdV-2 derived from the vaccination which probably protects the CaHV-1 positive and vaccinated dogs from progressive severity of disease.

Most of the CaHV-1-infected dogs in this study were adults. Apparently, they showed a lower clinical severity when compared with the infected puppies and younger dogs. CaHV-1 infection in adult to senior dogs was previously reported to cause mild respiratory symptoms that were localized and self-limited (Decaro et al., 2008). However, the evidence that CaHV-1 infection can induce a fatal systemic disease in adult dogs is increasing (Kawakami et al., 2010; Malone et al., 2010; Kumar et al., 2014). These findings can be explained by immunocompromised conditions, including infection, disease, under stress and aging, which then resulted in viral reactivation and shedding.

According to the necropsies of CaHV-1 infected puppies and positive PCR results of their bitches, the route of transmission could have occurred either by direct contact with vaginal secretion or transplacentally. Previously, CaHV-1 has been detected in the vaginal canal during whelping suggesting an infection risk to puppies (Kapil, 2015). The acute respiratory failure with disseminated hemorrhagic and necrotic foci in various organs, characterized by lesions in the puppies with CaHV-1 infection (Kapil, 2015) is consistent with the results of this study that showed massive widespread necrohemorrhagic lesions in the lung, liver, spleen and lymph nodes. In addition, damage to the respiratory epithelium and exfoliating of tissue debris that corresponded to tubular necrosis of the kidneys were also described. Some of the disseminated necrosis in several organs might have resulted from severe hypoxia due to viral pneumonia (Kumar et al., 2014). Compared with herpesvirus infection in other animals, most clinical signs after infection were involving respiratory systems according to its host cellular tropism and host restrictive property (Gaskell and Willoughby, 1999). However, other clinical signs affecting other organs were reported such as ocular diseases in feline herpesvirus-1 (FHV-1) infection (Gaskell and Willoughby, 1999), cardiac injuries in calves with bovine herpesvirus-1(BHV-1) infection (Hanedan et al., 2015), cauliflower-like mass at skin and genital tracts in dolphins with bottlenose dolphin herpesvirus (TTHV) infection (van Elk et al., 2009), and abortion with

encephalomyelitis in equine herpesvirus-1 (EHV-1) infection (Wozniakowski and Samorek-Salamonowicz, 2015).

Typically, the diagnosis of a CaHV-1 infection is performed by microscopic analysis based on the presence of intranuclear inclusion bodies in a variety of epithelial cells, such as bronchial and renal epithelia, hepatocytes, myocytes and ganglia cells, and accompanied with massive focal necrosis and hemorrhaging of the internal organs. However, less specific alterations in puppies and other systemic disease involvements in older animals leads to diagnostic ambiguities and requires a specific method to confirm the CaHV-1 infection, including immunohistochemistry, immunofluorescence, virus isolation, electron microscopy, and molecular assays (Schulze and Baumgärtner, 1998; Kumar et al., 2014; Kapil, 2015). Typically, CaHV-1 infections are difficult to confirm using immunohistochemistry, which has a lower sensitivity than PCR (DeBiasi et al., 2002), because of the instability and fragility of the virus in storage(Mills et al., 2013). However, most CaHV-1-infected PCR-positive dogs presented with disseminated necrotic and hemorrhagic lesions in the absence of herpesviral inclusion bodies (Kapil, 2015), which was in agreement with this study. Moreover, a cross-sectional study of 57 dead puppies in Denmark revealed that 22.8% of the dead puppies were positive for CaHV-1 infections when assayed by real-time PCR (qPCR), but the analysis by histological lesions were not consistent and the *in situ* hybridization results were mostly negative, except for one sample that contained a few positive cells (Larsen et al., 2015).

Several serological investigations have revealed the prevalence of CaHV-1 infections in household and colony breeding dogs in various countries at different time points, such as USA (12.8% during 2003–2004) (Carmichael et al., 1965), Italy (27.9%, assuming before 1998) (Sagazio P et al., 1998), Netherlands (39.3% during 1997–1998) (Rijsewijk et al., 1999), Belgium (45.8% in 2000) (Ronsse et al., 2002) and England (88% in 1994) (Reading and Field, 1999b). In Asia, a CaHV-1 serological survey was first reported in Korea and ranged from 18–70% depending on the region (Seo et al., 1994). In Japan, PCR-based CaHV-1 detection showed 12.8% positive CaHV-1 infections in inpatient dogs that presented with nosocomial infections during hospitalization and caused acute tracheobronchitis (Kawakami et al.,

2010).Therefore, this investigation increased the data on CaHV-1 prevalence in Asia using molecular and pathological characterizations in Thailand's dogs. Since 2003, a commercial inactivated subunit vaccine against CaHV-1 has been available in Europe and is recommended to use in pregnant dams (Poulet et al., 2001), but it is still unlicensed in several countries, including Thailand. Therefore, veterinarians should be aware of CaHV-1 infection levels and symptoms. A rapid and reliable genetic-based technique, such as PCR, might facilitate the ante-mortem diagnosis and prevent the CaHV-1 transmission in apparently clinically healthy animals.

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2.3 CROSS-SECTIONAL INVESTIGATION AND RISK FACTOR ANALYSIS OF COMMUNITY-ACQUIRED AND HOSPITAL-ASSOCIATED CANINE INFECTIOUS RESPIRATORY DISEASE COMPLEX

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ABSTRACT

Canine infectious respiratory disease complex (CIRDC) is associated with multiple factors. The transmission route can be via community-acquired infection (CAI) or hospital-associated infection (HAI), but the variable factors within these two routes are not well described. This study aimed (i) to investigate a cross-sectional prevalence of canine respiratory viruses including influenza (CIV), parainfluenza (CPIV), distemper (CDV), respiratory coronavirus (CRCoV), adenovirus-2 (CAdV-2) and herpesvirus (CaHV-1) in respiratory-disease dogs using multiplex PCR assays, and (ii) to analyze the possibly related risk factors. Two hundred and nine respiratory illness dogs consisted of 133 CAI and 76 HAI dogs were studied. Both nasal (NS) and oropharyngeal (OS) swabs were sampling from each dog. The respiratory viruses were detected by multiplex PCRs. Essential signalments, clinical signs, vaccination status, and route of transmission were recorded for further analysis. All six viruses were detected in both groups with CIV and CRCoV being predominantly found. Only CDV was significantly more prevalent in CAI than HAI dogs but not for the others. Multiple infections were found in 81.2% and 78.9% of CAI and HAI, respectively. Co-detection of CIV and CRCoV was significantly associated among study groups. Moreover, the clinical severity level was notably related to age of infected dogs, neither vaccination status, sex nor transmission route were noted.

INTRODUCTION

Canine infectious respiratory disease complex (CIRDC) is a major cause of respiratory illness and can be fatal in dogs (Buonavoglia and Martella, 2007). It is a complex condition, involving multifactorial etiologies. Overcrowding, stress, age and other underlying factors result in interference with the dog's immune response and play a role in disease predisposition (Erles et al., 2004; Schulz et al., 2014). Infectious agents serve as a key factor to promote a severity of clinical symptom. The viruses that are commonly associated with CIRDC are canine parainfluenza (CPIV), canine distemper (CDV), canine adenovirus type 2 (CAdV-2), canine herpesvirus 1 (CaHV-1), canine respiratory coronavirus (CRCoV) and canine influenza virus (CIV) (Erles et al., 2003; Erles et al., 2004; Crawford et al., 2005; Buonavoglia and Martella, 2007). During the last decade, novel viruses such as canine pneumovirus (CnPnV) and pantropic canine coronavirus (CCoV) (Buonavoglia et al., 2006; Renshaw et al., 2010), which induce respiratory problems in dogs have also been discovered. In addition, canine bocavirus (CBoV) and canine hepacivirus have been detected in the respiratory tract samples of dogs showing respiratory illness, but the pathogenic role of those viruses are underdetermined (Kapoor et al., 2011; Kapoor et al., 2012c). Although new viruses have emerged, the common CIRDC-associated viruses are still important contributors to respiratory diseases. Additionally, current core vaccines that are routinely used in dogs prevent some CIRDC viruses, such as CPIV, CDV and CAdV-2, but not CaHV-1, CRCoV and CIV. This is probably affecting in the virus spreading.

Hospital-associated infection (HAI) has recently been described as an "ever-present risk" (Weese and Stull, 2013). The current literature includes reported outbreaks of CPIV and CaHV-1 in animal healthcare facilities, where these nosocomial infections worsened the any ongoing disease and enhanced the morbidity and mortality rates (Kawakami et al., 2010; Weese and Stull, 2013). In addition, infections among communities are documented and serve as a source for disease dissemination. Community-acquired infections (CAIs) are those that are acquired without exposure to hospitals or with limited regular exposure with a health care center or both (Musher and Thorner, 2014). Currently, CAIs with CIRDC viruses are believed to have a

concomitant effect in respiratory disease (Kumagai et al., 2004; Buonavoglia and Martella, 2007; Priestnall et al., 2009; Mitchell et al., 2013; Viitanen et al., 2015).

The identification of multiple viral induced CIRDC involves the simultaneous rapid nucleic acid-based detection, typically by the reverse transcription polymerase chain reaction (RT-PCR) for RNA viruses and PCR for DNA viruses. These detection methods have led to an increased and better realization of the prevalence of CIRDC worldwide (Jeoung et al., 2013; Schulz et al., 2014; Piewbang et al., 2016). However, whether the severity of respiratory disease depends on the number of viral infections is equivocal. Although epidemiological evidence of CIRDC has been reported periodically, the classification among HAIs and CAIs has largely not been evaluated (Erles et al., 2004; Viitanen et al., 2015). A comprehensive understanding of the source of infection (HA or CA) and other associated risk factors may help guide the successful implementation of preventive strategies. To emphasize this point, this study focused on the associations between the prevalence of all six CIRDC viruses, source of infection and the possibly related risk factors of the dog's age, sex and vaccine status. Moreover, the relationship between clinical severity and number of viral infections was also evaluated in respiratory-ill dogs during 2013–2015 in Thailand.

MATERIALS AND METHODS

Study design

The prospective cross-sectional study was conducted from March 2013 to April 2015. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant institutional guides on the care and use of laboratory animals (Chulalongkorn University Animal Care and Use Committee, No. 1431005).

Study population

Two hundred and nine dogs with respiratory illness were included in the study. A nasal swab (NS) and an oropharyngeal swab (OS) were obtained from each dog. These respiratory illness dogs were obtained from the animal hospitals, private

clinics and animal shelters located at Central (n = 127), Eastern (n = 21), Southern (n = 49) and Western (n = 12) regions of Thailand. Any dog with respiratory problems associated with underlying cardio-pulmonary disease, functional or anatomical airway disease and neoplasia, based on physical examination and radiographic investigation, was excluded from the study. The history of hospital/clinic, shelter and pet grooming visits of the dogs was recorded and used to categorize the dogs into the CAI and HAI dog groups. The CAI group was comprised of dogs with respiratory illness and no history of veterinary hospital/clinic, shelter and pet grooming visits for at least the last 14 days prior to admission, while the HAI group was comprised of likewise symptomatic dogs that had a history of veterinary hospital/clinic, shelter or pet grooming visits within the last14 days.

Respiratory signs of the dogs were scored by a veterinarian on the date of sampling, as follows: 1, either nasal discharge or cough; 2, nasal discharge and cough; 3, nasal discharge or/ and cough with dyspnea; 4, evidence of bronchopneumonia based on auscultation or radiographs. The score was further grouped for the clinical severity level as mild (score 1 and 2), moderate (score 3) and severe (score 4). General signalment of age, breed, sex and vaccination status was systemically recorded for further interpretation.

Sample collection

The NS samples were collected by gently inserting a sterile rayon tipped applicator (Puritan®, Puritan Medical Product, ME, U.S.A.) into the nostril to a depth of approximately 2 cm and the OS samples were collected by rolling the swab onto the soft palate. The swabs were immersed in 0.5 ml of 1% sterile phosphate buffered saline (PBS) and stored at -80 °C until assayed.

Viral nucleic acid extraction and reverse transcription

Viral nucleic acids were extracted from the NSor OS samples (above) using the Viral Nucleic Acid Extraction Kit II (GeneAid, Xizhi, Taiwan) according to manufacturer's protocol, and then quantified and qualified using Nanodrop® Lite (Thermo Fisher

Scientific Inc., Massachusetts, U.S.A.) at an absorbance of 260 and 280 nm. The extracted nucleic acid was divided into two aliquots, one for two-stage RT-PCR for RNA viruses (CIV, CPIV, CDV and CRCoV) and another for direct PCR for DNA viruses (CAdV-2 and CaHV-1). For the first (RT) stage of the RT-PCR assay, 100 ng of total RNA was used as the template for RT to generate complementary DNA (cDNA) using the Omniscript® Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany). The cDNA and DNA were kept at -20 ºC until used for subsequent PCR amplifications.

Multiplex RT-PCR/PCR for RNA/DNA virus detection

Both multiplex RT-PCR (for CIV, CPIV, CDV and CRCoV) and multiplex PCR (for CAdV-2 and CaHV-1) were performed as previously reported (Piewbang et al., 2016; Piewbang et al., 2017). In addition, a first round of RT-PCR for CRCoV detection was conducted prior to performing the multiplex RT-PCR to augment the detection sensitivity. Specific oligonucleotide primers for CIV (M gene), CPIV (NP gene), CDV (NP gene), CRCoV (S gene), CAdV-1 (E3 gene), CaHV-1 (GB gene) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control were used. The PCR primer sequences, reaction mixture, assay conditions and amplicon visualization were performed as reported (Piewbang et al., 2016).

Statistical analysis

Descriptive analysis was used as the median for age. Data were evaluated using SPSS statistical analyser version 22.0 software (IBM Corp, New York, U.S.A). Differentiation of the CAI and HAI groups was compared using Fisher's exact and χ^2 tests. Clinical severity level against the demographic of infected dogs was performed using χ^2 tests. Prevalence of CIRDC viruses was calculated with a confidence interval (CI) of 95%. Associated respiratory score with respect to the CIRDC virus detections was evaluated using χ^2 tests for individual infection and logistic regression was evaluated in multiple infections. In all cases, a *P*-value of < 0.05 was considered statistically significant.

RESULTS

Clinical data of study population

The 209 dogs from four regions of Thailand including in this study were categorized into 133 CAI and 76 HAI dogs based on the presentation of their respiratory symptoms and history of hospital visits. Vaccination against CPIV, CDV and CAdV-2 was accounted for 32.3% (43/133) and 28.9% (22/76) dogs in the CAI and HAI groups, respectively. Unvaccinated CAI dogs were 56.4% (75/133) and unvaccinated HAI dogs were 59.2% (45/76). No history of vaccination was recorded in 15 (11.3%) of the CAI dogs and 9 (11.8%) of the HAI dogs.

The CAI group was comprised of 58.6% (78/133) male and 39.8% (53/133) female dogs, while the HAI group was 46.1% (35/76) male and 43.4% (33/76) female. Gender was not documented in two CAI and eight HAI group dogs. The age of the dogs ranged from 1 month to 14 years in the CAI group (median; 7 months) and from 0.5 month to 13 years in the HAI group (median; 7 months). Shi Tsu was the major breed in both the CAI (48.2%) and HAI (39.1%) groups. Nasal discharge served as a common respiratory sign in dogs from both groups, accounting for 78.2% (CAI group) and 78.9% (HAI group) of the dogs, followed by cough, dyspnea and bronchopneumonia as 66.2% (CAI) and 61.8% (HAI), 49.6% (CAI) and 53.9% (HAI), and 28.6% (CAI) and 30.3% (HAI), respectively. The presence of nasal discharge and cough were higher in the CAI dogs than the HAI ones, whereas dyspnea and pneumonia were high percentage in the HAI dogs.

Detection of respiratory viruses

Analysis by multiplex PCR assays revealed that most dogs in both groups were positive for at least one CIRDC virus (96.9% and 94.7% of dogs in the CAI and HAI groups, respectively). Among these six CIRDC viruses, CIV and CRCoV were common found in both groups (CIV; 57.9 for CAI, 60.5% for HAI and CRCoV; 62.4% for CAI, 59.2% for HAI), while CAdV-2 was the least detected (8.3% for CAI and 11.8% for HAI). The populations of other viruses in CAI and HAI groups were CPIV, CaHV-1 and CDV,

respectively. The CAI dogs had a significantly higher prevalence of CDV detection than the HAI dogs (P = 0.049; Table 6).

For multiple virus detections, the prevalence was 81.2% (108/133) in CAI dogs and 78.9% (60/76) in HAI dogs (Table 7.). The increased number of viral co-detection was similar in both groups and revealed that double viral detections were most frequently found. The frequency of multiple virus detections in the two groups decreased with increasing numbers of viruses, with no multiple detections of all six viruses being found in either the CAI or HAI dogs. There was no significant difference between the CAI and HAI dogs for multiple virus detections.

Table 6. Detection of CIRDC viruses in respiratory ill dogs between community-acquired infection (CAI) and hospital-associated infection (HAI) groups using multiplex PCR assays.

CIRDC virus	CAI[a]	HAI[a]	p-value
CIV	57.9 (77/133) 49.4-65.9	60.5 (46/76) 49.3-70.8	0.701
CPIV	39.1 (52/133) 31.2-47.6	32.9 (25/76) 23.4-44.1	0.371
CDV	35.3 (47/133) 27.7-43.8	22.4 (17/76) 14.4-33.3	0.049*
CRCoV	62.4 (83/133) 53.9-70.2	59.2 (45/76) 47.9-69.6	0.648
CAdV-2	8.3 (11/133) 4.5-14.3	11.8 (9/76) 6.1-21.2	0.398
CaHV-1	39.1 (52/133) 31.2-47.6	32.9% (25/76) 23.4-44.1	0.371

[a] Data are shown as the %, (number of infected dogs/totalnumber assayed) and (below) the 95% confidence limits,

*Statistically significant

Table 7. Detection of multiple CIRDC virus infections in respiratory ill dogs between community-acquired infection (CAI) and hospital-associated infection (HAI) groups using multiplex PCR assays.

Infection	CAI dogs[a]	HAI dogs[a]	*P* value
2 viruses	35.3 (47/133) 27.72-43.78	43.4 (33/76) 32.86–54.62	0.248
3 viruses	30.1 (40/133) 22.91–38.86	27.6 (21/76) 18.78–38.63	0.709
4 viruses	13.5 (18/133) 8.65–20.47	5.3 (4/76) 1.67–13.16	0.061
5 viruses	2.3 (3/133) 0.48–6.72	2.6% (2/76) 0.17–9.65	0.864
6 viruses	0	0	-

[a] Date are shown as the %, (number of infected dogs/total number assayed) and (below) the 95% confidence limits

When the variable demographic factors and single or multiple CIRDC virus detections were analyzed, main population was male and puppy in both groups. Most of infected dogs were unvaccinated. The common respiratory problem in both group were nasal discharge, cough and dyspnea; however, there was no association between the sex, age, vaccination status and respiratory signs against single or multiple CIRDC-virus detections (Table 8.). The variable demographic factors were also compared with clinical severity level, revealing most CIRD-infected puppies had more severity compared with other age groups. There was a statistically significant association

between the age of the dogs and clinical severity level (*P* = 0.012), with the exception of sex, vaccination status, type of affected dogs and number of viral detections (Table 9.). The respiratory score was compared with CIRD agents detected in both CAI and HAI (Table 10.). Most of CIRD positive dogs expressed respiratory score 3 and 4 in both CAI and HAI, accounted for 19.1% and approximately 11%, respectively. Moreover, double viruses of CIV and CRCoV was also predominantly detected in both groups with statistical association (*P* = 0.009, Table 10.).

Table 8. Characteristics of 209 respiratory ill dogs compared between the numbers of different CIRDC virus infections, as detected using multiplex PCR assays.

Characteristic	Number of Infections			*P* value
	Single CIRDC virus (n = 33) N (%) [95% CI]	Multiple CIRDC virus (n = 168) N (%) [95% CI]	Negative (n = 8) N (%) [95% CI]	
Sex				0.824
Male	18 (54.5) [40.7-73.6]	90 (53.6) [48.2-63.4]	5 (62.5) [30.4-86.5]	
Female	13 (39.4) [26.4-59.3]	71 (42.2) [36.7 -51.8]	2 (25.0) [6.3-59.9]	
N/A	2 (6.1) [0.68-20.6]	7 (4.2) [1.9-8.5]	1 (12.5) [<0.01-49.2]	
Age				0.527
Puppy	11 (33.3) [19.7-50.5]	58 (34.5) [27.7-42.0]	7 (87.5) [40.1-93.7]	
Growing	8 (24.2) [13.0-42.3]	49 (29.2) [22.9-36.7]	1 (12.5) [0.11-49.2]	
Adult	9 (27.3) [14.9-44.4]	28 (16.7) [11.8-23.2]	0 (0)	
Senior	5 (15.2) [6.4-32.2]	33 (19.6) [14.4-26.5]	0 (0)	
Vaccination				0.949
Yes	10 (30.3) [19.8-52.7]	53 (31.6) [27.9-43.0]	2 (25.0) [6.3-59.9]	
No	19 (57.6) [47.3-80.2]	98 (58.3) [57.0-72.1]	3 (37.5) [13.5-69.6]	
N/A	4 (12.1) [4.2-27.9]	17 (10.1) [6.3-15.7]	3 (37.5) [13.5-69.6]	
Clinical signs				
Nasal discharge	27 (81.8) [65.3-91.8]	129 (76.8) [69.8-82.6]	8 (100.0) [62.7-100]	0.526
Cough	23 (69.7) [52.5-87.5]	107 (63.7) [56.2-70.6]	5 (62.5) [30.38-86.5]	0.509
Dyspnea	18 (54.5) [68.6-93.8]	84 (51.5) [42.5-57.5]	5 (62.5) [30.38-86.5]	0.633
Bronchopneumonia	10 (30.3) [17.3-47.5]	46 (27.4) [21.8-34.6]	5 (62.5) [30.38-86.5]	0.732

[a]Age of the infected dogs: puppy < 3 months; growing > 3 month–1 y; adult > 1–5 y; senior > 5 y.

N/A: no data available

Table 9. Clinical severity levels grading of respiratory ill dogs associated with possible variable factors.

Characteristic	Clinical severity level[a]			*P* value
	Mild (n = 83) N (%) [95% CI]	Moderate (n = 75) N (%) [95% CI]	Severe (n = 51) N (%) [95% CI]	
Sex				0.775
Male	45 (54.2)[45.3-66.6]	43 (57.3)[48.2-70.3]	25 (49.0)[39.2-66.7]	
Female	35 (42.2)[33.9-55.3]	29 (38.7)[29.7-51.8]	22 (43.1)[33.3-60.8]	
N/A	3 (3.6) [0.8-10.5]	3 (4.0) [0.9-11.6]	4 (7.8) [2.6-19.0]	
Age[b]				0.012*
Puppy	26 (31.3)[22.3-41.9]	21 (28.0)[19.1-39.1]	29 (57.0) [42.3-68.8]	
Growing	25 (30.1)[21.6-41.1]	24 (32.0)[17.4-34.6]	9 (17.6)[9.54-31.0]	
Adult	17 (20.5)[13.1-30.5]	11 (14.7)[6.4-19.5]	9 (17.6) [9.54-31.0]	
Senior	15 (18.1) [11.3-28.1]	19 (25.3) [12.9-28.9]	4 (7.8) [2.6-19.4]	
Vaccination				0.845
Yes	26 (31.3)[24.9-45.9]	22 (29.3)[23.1-45.4]	17 (33.3)[25.7-53.4]	
No	49 (59.1)[54.0-75.1]	44 (58.7)[54.6-76.9]	27 (53.0)[46.6-74.3]	
N/A	8 (9.6)[4.7-18.1]	9 (12.0)[6.2-21.5]	7 (13.7)[6.5-26.0]	
Type[c]				0.988
CAI	53 (63.9)[53.1-73.4]	48 (64.0)[52.7-73.9]	32 (62.7)[49.0-74.7]	
HAI	30 (36.1)[26.6-46.9]	27 (36.0)[26.0-47.3]	19 (37.3)[25.3-51.0]	
Number of infections				0.590
Negative infection	1 (1.2)[<0.01-7.2]	2 (2.7)[<0.01-9.8]	5 (9.8) [3.8-21.4]	
Single infection	13 (15.7)[9.3-25.1]	10 (13.3)[7.2-23.0]	10 (19.6)[10.8-32.7]	
Double infection	32 (38.6)[28.8-49.3]	30 (40.0)[29.7-51.3]	18 (35.3)[23.6-49.1]	

Characteristic	Clinical severity level[a]			*P* value
	Mild (n = 83) N (%) [95% CI]	Moderate (n = 75) N (%) [95% CI]	Severe (n = 51) N (%) [95% CI]	
Triple infection	26 (31.3)[22.3-41.9]	22 (29.3)[20.2-40.5]	13 (25.5)[15.4-38.9]	
Quadruple infection	9 (10.8)[5.6-19.6]	9 (12.0)[6.2-21.5]	4 (7.8) [2.6-19.0]	
Quintuple infection	2 (2.4)[<0.01-8.9]	2 (2.7)[<0.01-9.8]	1 (2.0)[<0.01-11.3]	

[a] Clinical severity level: mild (score 1-2), moderate (score 3) and severe (score 4)

[b] Age of the infected dogs: puppy < 3 months; growing > 3 months–1 year; adult > 1–5 years; senior > 5 years.

[c] CAI: Community-acquired infection (n = 133); HAI: Hospital-associated infection (n = 76)

* Statistically significant.

Table 10. Association of CIRD agents with the clinical respiratory score.

	CAI[a]				HAI[a]				Total	*P*
	Clinical respiratory score				Clinical respiratory score					
	1	2	3	4	1	2	3	4		
Negative	0/20	6.1% (2/33)	2.5% (1/40)	2.5% (1/40)	7.7% (1/13)	0/16	8.3% (2/24)	4.3% (1/23)	3.8% (8/209)	0.474
CDV	0/20	3.0% (1/33)	0/40	0/40	0/13	0/16	0/24	0/23	0.5% (1/209)	0.947
CaHV-1	0/20	12.1% (4/33)	5.0% (2/40)	10.0% (4/40)	0/13	0/16	4.2% (1/24)	4.3% (1/23)	5.7% (12/209)	0.580
CIV	5.0% (1/20)	3.0% (1/33)	5.0% (2/40)	5.0% (2/40)	7.7% (1/13)	6.3% (1/16)	0/24	4.3% (1/23)	4.3% (9/209)	0.662
CPIV	5.0% (1/20)	0/33	0/40	0/40	0/13	6.3% (1/16)	4.2% (1/24)	8.7% (2/23)	2.4% (5/209)	0.134
CRCoV	0/20	0/33	5.0% (2/40)	2.5% (1/40)	0/13	12.5% (2/16)	0/24	4.3% (1/23)	2.9% (6/209)	0.086
CAdV-2+CaHV-1	0/20	0/33	0/40	2.5% (1/40)	0/13	6.3% (1/16)	4.2% (1/24)	0/23	1.4% (3/209)	0.220
CDV+CAdV-2	0/20	0/33	0/40	0/40	0/13	0/16	0/24	4.3% (1/23)	0.5% (1/209)	0.058
CDV+CaHV-1	0/20	0/33	2.5% (1/40)	2.5% (1/40)	0/13	0/16	4.2% (1/24)	0/23	1.4% (3/209)	0.568
CDV+CRCoV	0/20	0/33	5.0% (2/40)	7.5% (3/40)	0/13	0/16	4.2% (1/24)	0/23	2.9% (6/209)	0.641
CIV+CDV	0/20	0/33	2.5% (1/40)	2.5% (1/40)	0/13	0/16	0/24	0/23	1.0% (2/209)	0.865
CIV+CaHV-1	5.0% (1/20)	0/33	0/40	5.0% (2/40)	7.7% (1/13)	6.3% (1/16)	8.3% (2/24)	0/23	3.3% (7/209)	0.282
CIV+CPIV	0/20	6.1% (2/33)	2.5% (1/40)	0/40	7.7% (1/13)	0/16	0/24	4.3% (1/23)	2.4% (5/209)	0.404
CIV+CRCoV	5.0% (1/20)	12.1% (4/33)	20.0% (8/40)	7.5% (3/40)	46.2% (6/13)	25.0% (4/16)	20.8% (5/24)	8.7% (2/23)	15.8% (33/209)	0.009*
CPIV+CDV	10.0% (2/20)	3.0% (1/33)	2.5% (1/40)	2.5% (1/40)	0/13	0/16	0/24	0/23	2.4% (5/209)	0.601
CPIV+CaHV-1	0/20	6.1% (2/33)	0/40	0/40	7.7% (1/13)	0/16	0/24	4.3% (1/23)	1.9% (4/209)	0.282
CPIV+CRCoV	15.0% (3/20)	0/33	0/40	2.5% (1/40)	0/13	0/16	0/24	4.3% (1/23)	2.4% (5/209)	0.642
CRCoV+CAdV-2	0/20	0/33	0/40	0/40	0/13	0/16	0/24	4.3% (1/23)	0.5% (1/209)	0.057
CRCoV+CaHV-1	0/20	3.0% (1/33)	2.5% (1/40)	5.0% (2/40)	0/13	0/16	0/24	4.3% (1/23)	2.4% (5/209)	0.592
CDV+CAdV-2+CaHV-1	5.0% (1/20)	0/33	0/40	0/40	7.7% (1/13)	0/16	0/24	0/23	1.0% (2/209)	0.077
CDV+CRCoV+CaHV-1	0/20	0/33	0/40	5.0% (2/40)	0/13	0/16	0/24	0/23	1.0% (2/209)	0.865
CIV+CDV+CaHV-1	5.0% (1/20)	0/33	2.5% (1/40)	0/40	0/13	6.3% (1/16)	4.2% (1/24)	0/23	1.9% (4/209)	0.399

	CAI[a]				HAI[a]				Total	*P*
	Clinical respiratory score				Clinical respiratory score					
	1	2	3	4	1	2	3	4		
CIV+CDV+CRCoV	5.0% (1/20)	3.0% (1/33)	5.0% (2/40)	10.0% (4/40)	7.7% (1/13)	6.3% (1/16)	4.2% (1/24)	0/23	5.3% (11/209)	0.677
CIV+CPIV+CAdV-2	0/20	3.0% (1/33)	2.5% (1/40)	0/40	0/13	0/16	0/24	0/23	1.0% (2/209)	0.865
CIV+CPIV+CDV	0/20	0/33	0/40	0/40	0/13	0/16	0/24	4.3% (1/23)	0.5% (1/209)	0.058
CIV+CPIV+CRCoV	5.0% (1/20)	6.1% (2/33)	2.5% (1/40)	10.0% (4/40)	0/13	12.5% (2/16)	8.3% (2/24)	8.7% (2/23)	6.7% (14/209)	0.563
CIV+CRCoV+CAdV-2	0/20	3.0% (1/33)	2.5% (1/40)	0/40	0/13	0/16	0/24	0/23	1.0% (2/209)	0.865
CIV+CRCoV+CaHV-1	10.0% (2/20)	6.1% (2/33)	0/40	5.0% (2/40)	0/13	6.3% (1/16)	0/24	13.0% (3/23)	4.8% (10/209)	0.146
CPIV+CDV+CAdV-2	0/20	0/33	5.0% (2/40)	0/40	0/13	0/16	4.2% (1/24)	0/23	1.4% (3/209)	0.568
CPIV+CDV+CaHV-1	0/20	3.0% (1/33)	2.5% (1/40)	0/40	0/13	0/16	0/24	4.3% (1/23)	1.4% (3/209)	0.542
CPIV+CRCoV+CAdV-2	0/20	0/33	0/40	0/40	0/13	0/16	4.2% (1/24)	0/23	0.5% (1/209)	0.069
CPIV+CRCoV+CaHV-1	5.0% (1/20)	6.1% (2/33)	2.5% (1/40)	2.5% (1/40)	0/13	0/16	4.2% (1/24)	0/23	2.9% (6/209)	0.641
CIV+CDV+CRCoV+CaHV-1	5.0% (1/20)	3.0% (1/33)	2.5% (1/40)	0/40	0/13	0/16	4.2% (1/24)	0/23	1.9% (4/209)	0.645
CIV+CPIV+CAdV-2+CaHV-1	0/20	0/33	2.5% (1/40)	0/40	0/13	0/16	0/24	0/23	0.5% (1/209)	0.947
CIV+CPIV+CDV+CRCoV	0/20	9.1% (3/33)	7.5% (3/40)	2.5% (1/40)	0/13	6.3% (1/16)	0/24	0/23	3.8% (8/209)	0.449
CIV+CPIV+CRCoV+CAdV-2	0/20	0/33	0/40	2.5% (1/40)	0/13	0/16	0/24	0/23	0.5% (1/209)	0.947
CIV+CPIV+CRCoV+CaHV-1	10.0% (2/20)	3.0% (1/33)	2.5% (1/40)	0/40	0/13	0/16	0/24	0/23	1.9% (4/209)	0.686
CPIV+CDV+CRCoV+CaHV-1	5.0% (1/20)	0/33	0/40	2.5% (1/40)	0/13	0/16	0/24	4.3% (1/23)	1.4% (3/209)	0.542
CPIV+CRCoV+CAdV-2+CaHV-1	0/20	0/33	0/40	0/40	0/13	0/16	4.2% (1/24)	0/23	0.5% (1/209)	0.375
CIV+CPIV+CDV+CRCoV+CAdV-2	0/20	0/33	2.5% (1/40)	0/40	0/13	0/16	4.2% (1/24)	0/23	1.0% (2/209)	0.375
CIV+CPIV+CDV+CRCoV+CaHV-1	0/20	0/33	2.5% (1/40)	2.5% (1/40)	0/13	0/16	0/24	4.3% (1/23)	1.4% (3/209)	0.542
	9.6% (20/209)	15.8% (33/209)	19.1% (40/209)	19.1% (40/209)	6.2% (13/209)	7.7% (16/209)	11.5% (24/209)	11.0% (23/209)		

[a] Data are shown as the %, (number of infected dogs/total number assayed); CIV, canine influenza; CPIV; canine parainfluenza; CDV, canine distemper; CRCoV, canine respiratory coronavirus; CAdV-2; canine adenovirus-2; CaHV-1, canine herpesvirus 1

*Statistically significant; overall *P* value for differences among the infected categories (chi-square test for single infection and logistic regression for coinfection).

The weather in Thailand was examined for any associated risk of CIRCD prevalence in the period between March 2013 and April 2015. The average humidity, temperature and amount of rainfall were compared with the number of CIRDC infected dogs (Fig 8). The highest proportion of CIRDC virus infected dogs occurred during the rainy season (July–October) in 2014 (66.1%), then followed by the winter (November 2014 to February 2015; 16.5%) and summer (March–April 2015; 14.9%), respectively. However, this was not the case in the rainy season in 2013. The highest level of infections was in the period of a relatively high amount of rainfall (range 190.6–245.9 mm).

DISCUSSION

The CIRDC, a common respiratory disease complex in dogs, is associated with environmental factors, individual host susceptibility and infectious pathogens, which they are primarily viruses (Buonavoglia and Martella, 2007; Priestnall et al., 2014). The CIRDC viruses are commonly detected in dogs with respiratory problems and are endemic in poor conditioned dogs, overcrowded shelters, hospitals and pet grooming centers (Erles et al., 2004; Kawakami et al., 2010; Weese and Stull, 2013; Schulz et al., 2014; Monteiro et al., 2016). A hospital associated infection compared with community infection is not well established. The epidemiology of CIRD infected dogs has been reporting in many years, but represented contrasting results (Erles et al., 2004; Schulz et al., 2014; Viitanen et al., 2015). In this study, we focused on the prevalence of CIRDC virus detections in terms of CAI and HAI that are supposed to be the factor of CIRDC infections. The HAI-diseases, especially respiratory tract infections, have been considered as a risk for nosocomial transmission, but they have not been investigated in clinically infected dogs (Kawakami et al., 2010; Weese and Stull, 2013). There has been increasing concern about infections due to

emergence of multidrug resistant pathogens as well as novel pathogens that are transmitted from other hosts (Weese and Stull, 2013).

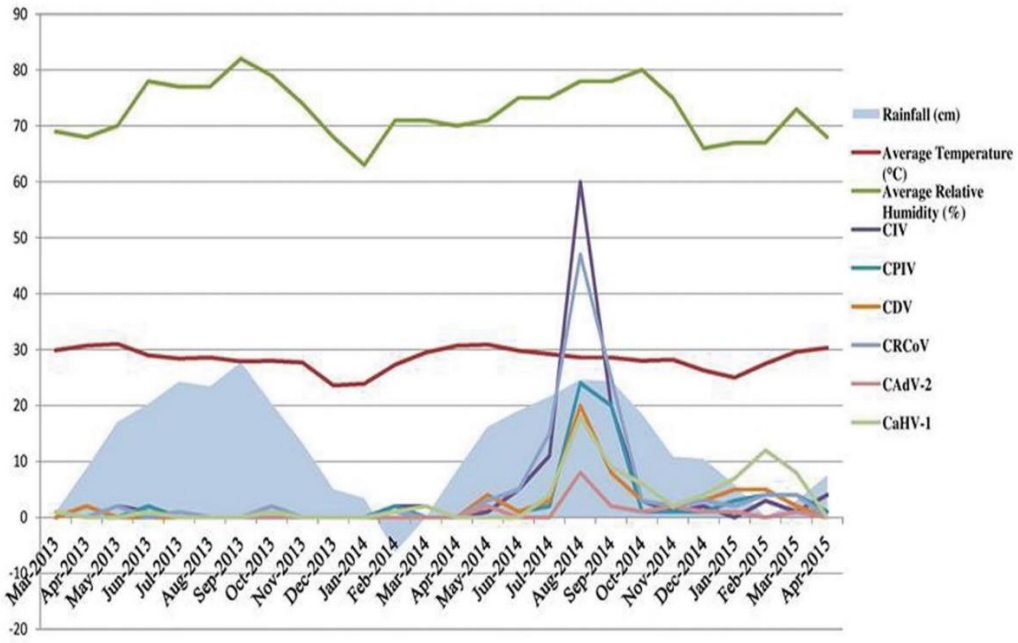

Figure 8. Detection of CIRDC viruses in 209 respiratory ill dogs, seasonal factors. Season was classified into the three periods of summer (March–June), rainy (July–October) and winter (November–February). Monthly percentage of CIRDC virus detections was compared with the monthly average temperature, relative humidity and amounts of rainfall during March 2013 to April 2015.

In this study, all six viruses were detected in both sample groups, suggesting that these viruses might either be presented or disseminated in both environments. Moreover, we found that CRCoV and CIV were highest prevalence in the CAI and HAI dogs, respectively. This finding was in contrast to previous observations found that CPIV was the most detected virus in CIRDC dogs (Erles et al., 2004; Schulz et al., 2014; Viitanen et al., 2015), although this might be influenced by various factors, including the different geography, vaccination strategies and hospital hygienic

management. This study showed that CDV was significantly detected in CAI dogs than that in HAI counterpart. In Thailand, there are many unvaccinated free-roaming dogs that could serve as a reservoir for CDV dissemination in CAI dogs (Posuwan et al., 2010; Radtanakatikanon et al., 2013).Meanwhile, other CIRDC viruses were detected in both the CAI and HAI dogs without any statistically significant difference in the frequency of occurrence between them. This is in accord with a recent study reported that there was no statistically significant difference in CPIV, CAdV-2 and CRCoV infections between dogs from private households and dogs from shelters or kennels (Schulz et al., 2014). Similar to other investigations focusing on HAI-derived CIRDC endemics, we found all six CIRDC viruses were detected in the HAI dogs (Kawakami et al., 2010; Weese and Stull, 2013). This might imply that these viruses already are endemic or spread in animal health care centers, such as hospitals or pet grooming centers.

The prevalence of CDV, CPIV, CAdV-2, CaHV-1 and CIV circulation in Thailand has been documented (Posuwan et al., 2010; Bunpapong et al., 2014; Piewbang et al., 2016; Piewbang et al., 2017), but not for that of CRCoV. This study, therefore, is the first report of CRCoV infected dogs in Thailand. The most commonly detected viruses were CIV and CRCoV (> 50%) in both CAI and HAI groups that is in contrast to previous reports that CPIV is the major CIRDC virus found in dogs with respiratory disease in Asia (Mochizuki et al., 2008; Posuwan et al., 2010)and Europe (Erles et al., 2004; Schulz et al., 2014; Viitanen et al., 2015; Decaro et al., 2016).

In the present study, 81.2% (108/133) of CAI and 78.9% (60/76) of HAI dogs were multiple virus detections, supporting the complex condition of this respiratory disease, which is often a co-infection rather than a single pathogen (Mochizuki et al., 2008; Schulz et al., 2014; Viitanen et al., 2015; Monteiro et al., 2016; Piewbang et al., 2016). We also observed that the majority of multiple CIRDC viruses were co-detected with either CIV, CRCoV, or both; however, this observation does not allow interpretation as to whether they are primary and/or secondary pathogens. The CRCoV and CIV usually induced mild clinical symptoms by interfering with the respiratory defense mechanism and so leading to super-infections with other pathogens (Chilvers et al., 2001; Buonavoglia and Martella, 2007; An et al., 2010b).

Moreover, co-infection of CIV and CRCoV may be synergistic and lead to severe tracheobronchitis (An et al., 2010b).

There were no significant differences in the sex, age and vaccination status of dogs between single and multiple virus detections. This finding need to be awareness for interpretation because of no significant difference between vaccinated- and unvaccinated dogs. The commercial vaccine used in Thailand against only CPIV, CDV and CAdV-2 but not for others. Thus, the multiple infections may be caused by the other viruses, which have no commercial vaccine available, and may affect the study results by allowing infection with other viruses in the vaccinated dogs. However, there were different levels of single and multiple virus detections between vaccinated and unvaccinated dogs. The vaccinated dogs had a lower proportion of both single and multiple detections compared with the unvaccinated dogs. Interestingly, there was significant difference in CIRD-affected age groups when compared with clinical severity level. CIRD-affected puppies had more severe clinical level compared with other age groups. Because host susceptibility such as premature immune response and unvaccinated dogs might be explained in this finding. However, there was no significant difference of clinical severity level between CAI and HAI dogs which is inconsistent with our hypothesis.

Significant association between CIRD agents and clinical respiratory scores was observed in double detection of CIV and CRCoV. This co-detection represented the highest proportion and most often found with other CIRD viruses. These observations showed that CIV and/or CRCoV can be either primary or secondary agents that commonly found co-infected with others; however, bacterial infection was not ruled out in this study. Many investigations have revealed that either viral or bacterial co-infection leads to an increased severity, but some studies have revealed no significant differences in the clinical severity between single and multiple infections (Cilloniz et al., 2012; Huijskens et al., 2014; Viitanen et al., 2015; Decaro et al., 2016).

Because CIRDC is caused by multi-factorial etiologies, changes in the environment, such as climate temperature and amount of rainfall, may affect the host susceptibility to the pathogens. The prevalence of CIRDC infections was seen to increase as the amount of rainfall increased and after the average temperature

declined (July–October 2014). Nevertheless, even when the rainfall amount and average temperature increased during January–April 2015 the prevalence of CIRDC infections still slightly increased. However, this finding lack of power to explain the relation of CIRDC detected dogs and any climate changes. Previous studies regarding respiratory viruses in climate zones and tropical countries have documented that infectious respiratory viruses, especially influenza, are usually observed during or immediately after the rainy season (Chow et al., 2006; Nguyen et al., 2007; Prachayangprecha et al., 2013). However, the CIRDC infection patterns were not represented during March–December 2013 because of the limited number of samples in this study. Other factors, such as the population size and density, geographic variation, natural phenomenon (such as El Niño) and the annual variability in the climate, might have affected the prevalence of infections (Shoji et al., 2011; Yang et al., 2011; Prachayangprecha et al., 2013).

Since healthy or asymptomatic dogs were not included in this study then the true prevalence of the CIRDC virus infections could not be assessed. Moreover, some of CIRDC virus such as CPIV, CAdV-2 and CRCoV could be detected from healthy dogs (Erles et al., 2004; Schulz et al., 2014; Viitanen et al., 2015), suggesting that some of CIRDC virus may not exhibit respiratory problem; however, individual host susceptibility should be concerned. It would be interesting to study healthy dogs, which might serve as an asymptomatic reservoir for CIRDC infections in the CAI group. Furthermore, the result of CIRDC virus detection by using PCRs form the respiratory swabs could not be totally implied that those dogs were “infected” but only a detection of represent CIRDC pathogens that might associate with respiratory problem. Additionally, samples from the North and North Eastern regions of Thailand were not collected for practical reasons and so the epidemiology could not be achieved in this study. Moreover, since we only focused on six viral pathogens associated with CIRDC, then the role of other pathogens, including known bacterial pathogens, such as *Bordetella bronchoseptica* and *Mycoplasma* sp., and novel viruses, such as CBoV and CnPnV, that might be implicated remains unknown.

CONCLUSIONS

All six respiratory viruses examined, and primarily CIV and CRCoV, were detected in both CAI and HAI dogs. The CIV and CRCoV were often detection with themselves or others and might increase clinical respiratory problem. Age may affect with clinical severity level. No difference in the types of CIRDC-associated viruses was found between the CAI and HAI groups, with the exception of the CDV-association with CAI dogs. The clinical severity of CIRDC infection was related to age of affected dogs, but not for multiple infections, type of affected dogs (CAI and HAI) and vaccination. Additionally, the variable respiratory signs and vaccination did not differ between single and multiple infections. An effect of climate change could not be ruled out for enhancing the CIRDC prevalence in this study.

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2.4 A NOVEL STRAIN OF CANINE BOCAVIRUS TYPE-2 INFECTION ASSOCIATED WITH INTESTINAL LESIONS REMINISCENT OF CANINE BOCAVIRUS TYPE-1 INFECTION.

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ABSTARCT

Bocaviruses are small non-enveloped DNA viruses belonging to the *Bocaparvovirus* genus of the *Parvoviridae* family and have been linked to both respiratory and enteric disease in humans and animals. To date, three bocaviruses, canine bocaviruses 1-3 (CBoV 1-3), have been shown to affect dogs with different disease manifestations reported for infected animals. We have used next generation sequencing to identify a novel strain of canine CBoV-2 (CBoV TH-2016) in a litter of pups that died in Thailand from acute disease, for which no causal etiological pathogen could be identified in routine screening assays. Analysis of the complete coding sequences of CBoV TH-2016 showed that this virus was most closely related to a strain previously identified in South Korea, with evidence of genetic recombination in the VP2 gene with a related strain from South Korea and Hong Kong. Use of quantitative PCR showed the presence of CBoV TH-2016 in several tissues, suggesting hematogenous virus spread, while only intestinal tissue was found to be positive by *in situ* hybridization. Histopathological lesions associated with CBoV TH-2016 infection in the enteric tract were similar to those previously considered to be pathognomonic for CBoV-1 infection. Therefore, this study provides novel insights in the pathogenicity of canine bocavirus infections and suggests that a novel recombinant CBoV-2, which was discovered from deceased pups showing negative for routine tests, may result in atypical syndrome. Although the specific cause of deaths of the pups was not established, a contributory role of enteric CBoV TH-2016 infection could not be excluded.

Keywords: Bocavirus, Dog, Genetic analysis, Pathology, Recombination

INTRODUCTION

The *Parvoviridae* family consists of small, non-enveloped, linear single stranded DNA viruses and is divided into two subfamilies: *Parvovirinae* infecting vertebrate hosts, and *Densovirinae* infecting arthropod hosts. To date members of three out of the eight *Parvovirinae* genera have been identified in dogs: canine parvovirus (CPV: genus *Protoparvovirus),* canine bocaviruses 1-3 (CBoV-1, -2, -3: genus *Bocaparvovirus)* and canine associated parvovirus (genus *Dependoparvovirus)* (Cotmore et al., 2014). CBoV-1, or as it was referred to initially, canine minute virus (CnMV), was first isolated in Germany in 1967 (Binn et al., 1970). Although most CBoV-1 infections in dogs appear to be subclinical, several studies have indicated a pathogenic role especially in unborn or young pups and old dogs. This infection is largely associated with respiratory, intestinal and reproductive problems (Macartney et al., 1988; Schwartz et al., 2002). The severity of clinical manifestations appears at least in part to be associated with CBoV-1 induced immunosuppression (Decaro et al., 2002; Manteufel and Truyen, 2008). A second canine *Bocaparvovirus* (CBoV-2) was identified in 2012 in association with canine respiratory diseases in a metagenomic study looking for hitherto unknown canine viruses (Kapoor et al., 2012b). The NS, NP and VP genes of CBoV-2 share less than 63, 62 and 64% amino acids identity with those of CBoV-1, respectively (Kapoor et al., 2012b). CBoV-2 infection also has been associated with massive enteritis in a litter of dogs with atrophied, shortened and fused villi, large crypt regeneration and large bone marrow and lymphoid atrophies (Bodewes et al., 2014). In addition, interstitial pneumonia has been reported to be a feature of CBoV-2 infection (Choi et al., 2015). Therefore, CBoV-2 infected dogs may present with respiratory and with enteric disease, as is also observed in human bocavirus (HBoV) infections. Finally, a third species of canine bocaparvovirus (CBoV-3) was identified in the liver of a dog co-infected with a recently identified circovirus (Li et al., 2013b). In the present study, we describe the genomic analysis of a novel CBoV-2 strain in puppies that had died in Thailand with intestinal histopathological findings, previously thought to be uniquely associated with CBoV-1 infection (Carmichael et al., 1994; Ohshima et al., 2010).

MATERIALS AND METHODS

Animals and post-mortem examination

Three 2-week old Welsh Corgi pups, which had died in Thailand with acute severe dyspnea, and hemoptysis, while their dam had remained asymptomatic, were subjected to routine postmortem examination at the Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University. Organs were immersed in 10% neutral buffered formalin, followed by processing into paraffin embedded blocks. Three micrometer thick sections in individual blocks were stained with hematoxylin and eosin (H&E) for histopathological examination. Fresh tissues, including lung, liver and tracheobronchial lymph nodes were collected aseptically and kept at -80 ºC for further investigation.

Routine diagnostic virology, bacteriology and immunohistochemistry

Fresh tissues were subjected to routine virological and bacteriological diagnostic assays. Samples were individually homogenized by using homogenizer grinds (FastPrep-24^{TM} 5G, MP Biomedicals, CA, U.S.A.) and centrifuged to collect the supernatant, which was used for nucleic acid isolation. Total viral nucleic acids were extracted using Viral Nucleic Acid Extraction Kit II (GeneAid, Taipei, Taiwan) according to manufacturer's protocol. Total cDNA was synthesized using 100 *n*g RNA as the template for complementary DNA (cDNA) synthesis using the Omniscript® Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany). A panel of virus associated canine infectious respiratory disease complex (CIRDC) detection was conducted using described multiplex PCR protocols to detect the presence of canine influenza virus, canine parainfluenza virus, canine distemper virus, canine respiratory coronavirus, canine adenovirus type 1 and 2 and canine herpesvirus type 1 (Piewbang et al., 2016). Moreover, pan-PCR assays specific for paramyxovirus, coronavirus and herpesvirus were carried out on the extracted nucleic acid as previously described with minor modifications (VanDevanter et al., 1996; Ksiazek et al., 2003; Tong et al., 2008). Small intestine and lymph node sections were subjected to immunohistochemistry (IHC)

using a mouse monoclonal anti-canine parvovirus antibody 1.B.450 (Abcam, Cambridge, UK).

For routine diagnostic bacteriology, the frothy tracheal exudate was cultured on standard agar under aerobic conditions (Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand).

Sample preparation for next-generation sequencing (NGS) and genome assembly

As the pups were suspected to have died from a respiratory problem, lung samples of three pups were individually prepared for deep sequencing using a modified sequence-independent single-primer amplification protocol (Allander et al., 2001). Briefly, after homogenization, RNA was extracted by Qiamp Viral RNA mini kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's recommendation. Extracted RNA was then transcribed to cDNA using a mixture of random and non-ribosomal hexamers with SuperScript IV (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) (Endoh et al., 2005), followed by Klenow reaction. Samples were randomly amplified using described primers and Taq polymerase (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.)(Allander et al., 2001) and PCR products were purified. Final DNA concentrations were measured using PicoGreen (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.). After samples were prepared, a DNA library was constructed following NexteraXT protocol (Illumina, San Diego, CA, U.S.A.) and deep sequenced on an Illumina MiSeq system using MiSeq Reagent kit V3 (300x2 cycles). Raw reads were initially screened by an *in house* metagenomics pipeline to identify viral reads. Trimmomatic (V0.36) was used for quality based trimming and adapter removal. The passed and processed reads were mapped to the full viral NCBI GenBank DNA and Peptide database using Bowtie 2 V2.2.9 for the DNA mapping and Pauda V1.0.1 for translation and amino acid mapping (Langmead and Salzberg, 2012; Huson and Xie, 2014).Species were ranked according to the number of reads mapped to each species, and read positions of the top findings were visualized for further selection of biologically reasonable taxa. Reference assembly was performed with CLC Genomics Workbench 9.0.

Canine bocavirus-2 specific PCR

Upon identification of CBoV-2 sequences, specific CBoV-2 primers were designed based on sequences obtained from NGS to obtain a complete consensus sequence (Table 11.). PCR reactions with a total volume of 50 µL were prepared as follows: 5x Phusion HF Buffer, 10 mM of dNTP, 1U of Phusion DNA polymerase (New England Biolab, Ipswich, MA, U.S.A.), 10 *µ*M final concentration of each primer, and 2 *µl* of template. Conditions for CBoV-2-specific PCR were: initial denaturation at 98 ºC for 30 sec, followed by 45 cycles of 98 ºC for 20 sec, 50 ºC for 30 sec, 72 ºC for 1 min and a final extension at 72 ºC for 7 min. The PCR products were run on a 1% agarose gel and amplified products were purified and Sanger sequenced (Eurofins Genomics, Munich, Germany).

Table 11. CBoV-2 specific primers used for complete genome sequencing.

Genome position[a] (nucleotide)	Sequence (5'-3')	Direction	Product size (bp)
112-131	CTWGTGGYYKGTTAATGTAT	Forward	791
882-902	GCAGGATGGTACAAATCTCGG	Reverse	
1035-1054	CTCACTTTGCCTGYTCTGGT	Forward	839
1854-1873	GTACACGTCGTGATTGGTAC	Reverse	
1892-1912	ACCACTACTATGGTTCACGCT	Forward	790
2661-2681	TTGATTGAAGACCTCCATCGG	Reverse	
2668-2687	AGGTCTTCAATCAACACCGC	Forward	670
3321-3337	CCTTTGTATCCGCGAGA	Reverse	
3321-3337	TCTCGCGGATACAAAGG	Forward	942
4244-4262	TCTGCCTGTGGAATGGCGT	Reverse	
4283-4301	CCAGACGATACTTTCCTAC	Forward	876
5143-5158	CCGTTTWGTGGGCATG	Reverse	

[a] Based on the CBoV TH-2016 genome.

Genome analyses

The newly obtained CBoV-2 sequence from Thailand (GenBank accession No.MG025952) was compared to those of other strains of CBoV-2 using MAFFT alignment version 7 (http://mafft.cbrc.jp/alignment/server/). Phylogenetic analyses were carried out with MEGA 7 (Kumar et al., 2016). A phylogenetic tree was constructed using neighbor-joining method with GTR+G+I as best-fit model of nucleotide substitution according to Bayesian Information Criterion. Bootstrap analysis was performed using 1000 replicates. Pairwise distance of CBoV-2 genome, NS1, NP, VP1/2 and ORF4 were calculated using BioEdit. Genetic recombination was analyzed by using similarity plot and bootscan tools in SimPlot software package version 3.5.1 modeled with a window size 200 bp and step size 20 bp (Lole et al., 1999).

Screening for CBoV-1 and CBoV-2

To distinguish between CBoV-1 and CBoV-2 infection, nucleic acids of fresh lung, liver and lymph node tissue samples were extracted as described previously. DNA from formalin-fixed paraffin-embedded (FFPE) containing small intestine tissue was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen GmbH, Hilden, Germany) following manufacturer's recommendations. The extracted DNA was tested with CBoV-2 specific primers (forward: GCTGTACGGATGTGTGAA; reverse: CAGACACTTGGCCTGCTCTA) (Bodewes et al., 2014). For CBoV-1, three sets of specific primers were used as described previously; CBoV-1_596+: AACGCGATTTGCACCTTCAT; CBoV-1_1113-: CATCAAACATTTCTCCGGCA (Choi et al., 2016); CBoV-1_3514+: GTGGTATGCACCTATATACAACGGAC; CBoV-1_4765-: GATGGAACTCTGCCTATGTCGCATCCG(Mochizuki et al., 2002); CBoV-1_4376+: AGGACCATCGCTTGGATACATT; CBoV-1_4445-: TACTGGTCCGAGGGCTTGTT (Sun et al., 2009), and designed primers obtaining from alignment of CBoV-1 sequences available in GenBank as CBoV-1_401+: TCTCGATGATCCATCCGTGT; CBoV-1_491-: GGAATCAGGTCCA TGTGTCTC, were used for CBoV-1 detection. Synthetic CBoV-1 sequence was retrieved from consensus NS1 regions of CBoV-1 strains available in

GenBank and used as positive control for PCR. The reactions consisted of 5x OneTaq Buffer, 10 mM of dNTP, 1U Hot start Taq polymerase (New England Biolab, Ipswich, MA, U.S.A.), 10 μM final concentration of each primer, and 2 μl of template. The PCR conditions were as follows: initial denaturation at 94 ºC for 5 min, followed by 45 cycles of 94 ºC for 30 sec, 50 ºC for 30 sec, 72 ºC for 30 sec and a final extension at 72 ºC for 10 min. The PCR products were run on a 2% agarose gel and all positive samples were Sanger sequenced.

To estimate the amount of CBoV-2 DNA in different organs, a SYBR Green based quantitative PCR (qPCR) (Brilliant III Ultra-Fast SYBR® Green qPCR, Agilent, Santa Clara, CA, U.S.A.) was performed in the AriaMx Real-time PCR system. The reaction comprised of 250 nM of each CBoV-2 specific primer (forward: GCTGTACGGATGTGTGAA; reverse: CAGACACTTGGCCTGCTCTA), 2 µl of template, and 1X of SYBR Green mix. The PCR conditions were as follows: 95 ℃ for 3 min followed by 40 cycles of 95 ℃ for 10 sec, 50 ℃ for 10 sec, and 60 ℃ for 10 sec. The amplicon melting temperature profile consisted of 95 ℃ for 30 sec, 60 ℃ for 30 sec and 95 ℃ for 30 sec. A positive amplicon was expected to melt at 82.5 ℃. An output Ct value was used to estimate the amount of virus presence.

In situ hybridization and transmission electron microscopy

To confirm the presence of CBoV-2, *in situ* hybridization (ISH) was performed on FFPE tissues, including heart, trachea, lung, liver, gall bladder, spleen, kidney, pancreas, small intestine and lymph nodes. Probes covering 124 bp of the NS1 gene of the canine bocavirus (GenBank Accession number KF771828; nt 1490-1513) were used as described previously.(Bodewes et al., 2014) Briefly, FFPE sections were de-paraffinized using xylene, then hydrated in graded ethanol, and washed in diethyl pyrocarbonate (DEPC)-treated water. For proteolytic digestion of samples, 1µg/ml proteinase K (Roche Diagnostics, Basel, Switzerland) was applied. Following post-fixation, acetylation and pre-hybridization, an overnight incubation with 500 ng/100 µl sense and antisense probe, respectively, at 52 ºC was performed. For detection, an anti-DIG antibody (diluted 1:200) conjugated with alkaline phosphatase (Roche

Diagnostics, Basel, Switzerland) was used in combination with nitrobluetetrazoliumchloride (NBT, Sigma-Aldrich, St. Louis, MO, U.S.A.) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, X-Phosphate, Sigma-Aldrich, St. Louis, MO, U.S.A.) as substrates. Purple precipitates with a clear, cellular association were considered positive. A known CBoV-2 positive sectionwas used as a positive control (Bodewes et al., 2014). A non-probe incubation served as a negative control.

Transmission electron microscopy for demonstration of virus particles within intranuclear inclusions of enterocytes was performed using the pop-off technique as described (Lehmbecker et al., 2014).

RESULTS

Gross- and histopathology

Gross pathological examination of the three carcasses did not show pathology in respiratory tissues, apart from nasal discharge with frothy and bloody tracheal fluid, multifocal discolorations of lung lobes, slightly blunted edges of liver and spleen, and brownish mucus with curd milk in stomachs and small intestines. Microscopic examination revealed multiple eosinophilic intranuclear inclusion bodies within enterocytes of the small intestine (Fig. 9), a pathological finding previously considered to be pathognomonic for CBoV-1 infection. One of the animals showed mild to moderate, multifocal infiltration of the small intestinal mucosa with neutrophils and low to moderate numbers of intraluminal nematodes. No atrophy, blunting or fusion of villi as has been reported for CBoV-2 infection in previous study (Bodewes et al., 2014). Despite variable degrees of acute alveolar edema and emphysema, lungs and trachea displayed no significant lesions such as inflammation (Fig. 10.).

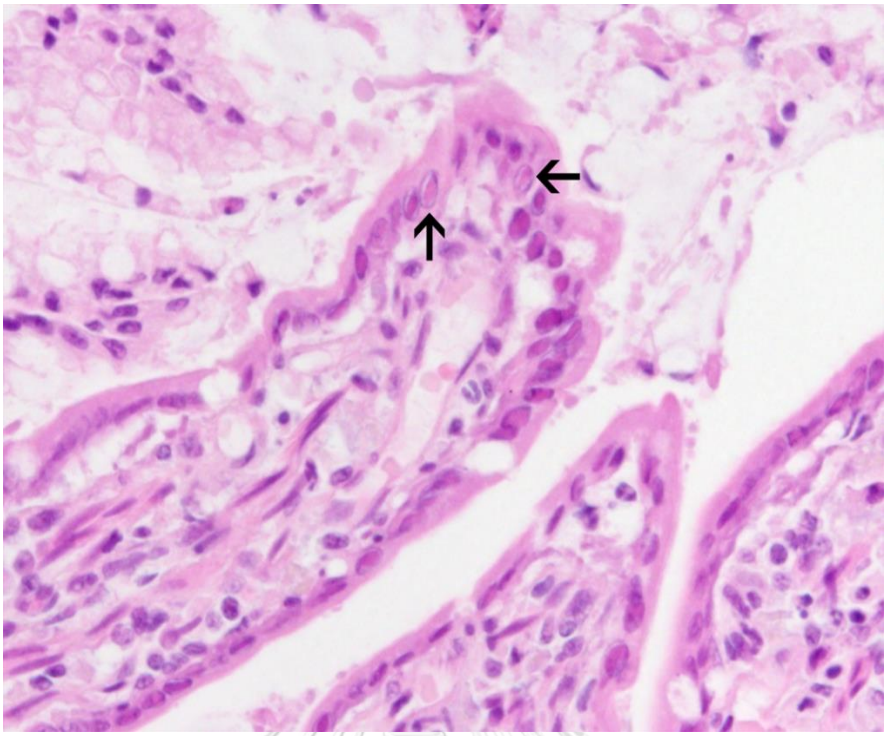

Figure 9. Canine bocavirus-2 infection, small intestine, dog. Multiple intranuclear inclusion bodies (INIB) are presented within enterocytes (arrows). Hematoxilin and eosin (H&E).

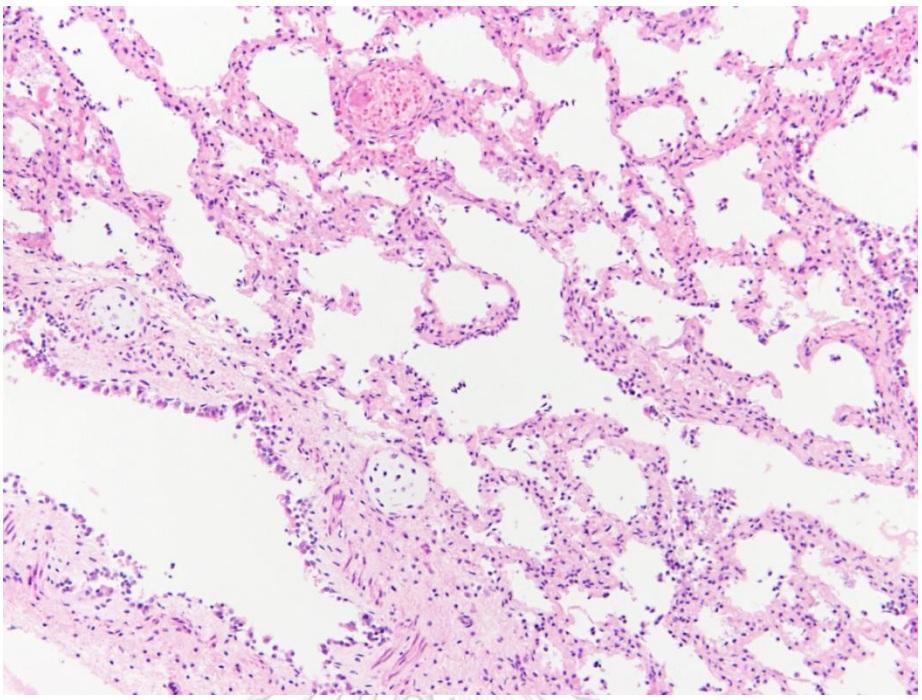

Figure 10. Canine bocavirus-2 infection, lung, dog. No inflammatory lesions are present within the lung. H&E.

Detection of CBoV-2 by NGS

Application of multiplex and pan-virus family PCR assays did not identify a causal viral etiology, while no CPV antigen could be detected in small intestine by immunohistochemistry. Apart from non-pathogenic *Aeromonas hydrophila* and *Klebsiella sp.* no bacteria were cultivated from tracheal fluid. Analyses of NGS data obtained from the *in house* metagenomics pipeline identified the presence of CBoV-2 (66, 36, 58 reads) in lung samples from all three pups. Approximately 0.005% of reads were mapped to CBoV-2 genome. A reference assembly was performed using reads from all three CBoV-2 positive puppies to construct a consensus viral genome. However, as gaps were still present in this sequence, multiple conventional PCRs were performed using primers designed for the newly identified CBoV-2, named CBoV TH-

2016 (GenBank Accession No. MG025952). A 5,126-bp nearly complete genome sequence was recovered, which comprised the three main open reading frames (ORFs). ORF1 encoded the overlapping nonstructural proteins (NS), NS1 (nt 226-2607; 794 amino acids) and NS2 (nt 226-2137 and nt 2212-2607; 637 and 132 amino acids). ORF2 encoded the overlapping viral capsid proteins VP1 (nt 2943-5060; 706 amino acids) and VP2 (nt 3357-5060; 568 amino acids). ORF3 encoded the nucleoprotein (NP) (nt 2372-2959; 196 amino acids). Furthermore, ORF4 (nt 2173-2607), was also detected downstream the NS1 in this consensus sequence (Fig. 11). Phylogenetic analyses of the complete CBoV-2 genomes revealed that CBoV TH-2016 is most closely related to a strain from South Korea (GenBank accession No. KP281718; 93.3% pairwise nucleotide and 87.8% amino acid identity) (Fig. 12). However, the phylogenetic tree based on VP1/2 and NS1 sequences revealed that CBoV TH-2016 grouped to clade of South Korea and Hong Kong strains, respectively (Figs. 13-14). Additionally, a deletion of 18 nucleotides was present in the VP1/2 gene (Fig. 15). The pairwise distances of NS1/2, NP, VP1/2, and ORF4 between CBoV TH-2016 and other CBoV-2 strains are shown in Table 12. On the basis of these data, CBoV TH-2016 should be considered a novel strain of CBoV-2 rather than a novel canine bocavirus species (Bodewes et al., 2014; Choi et al., 2016).

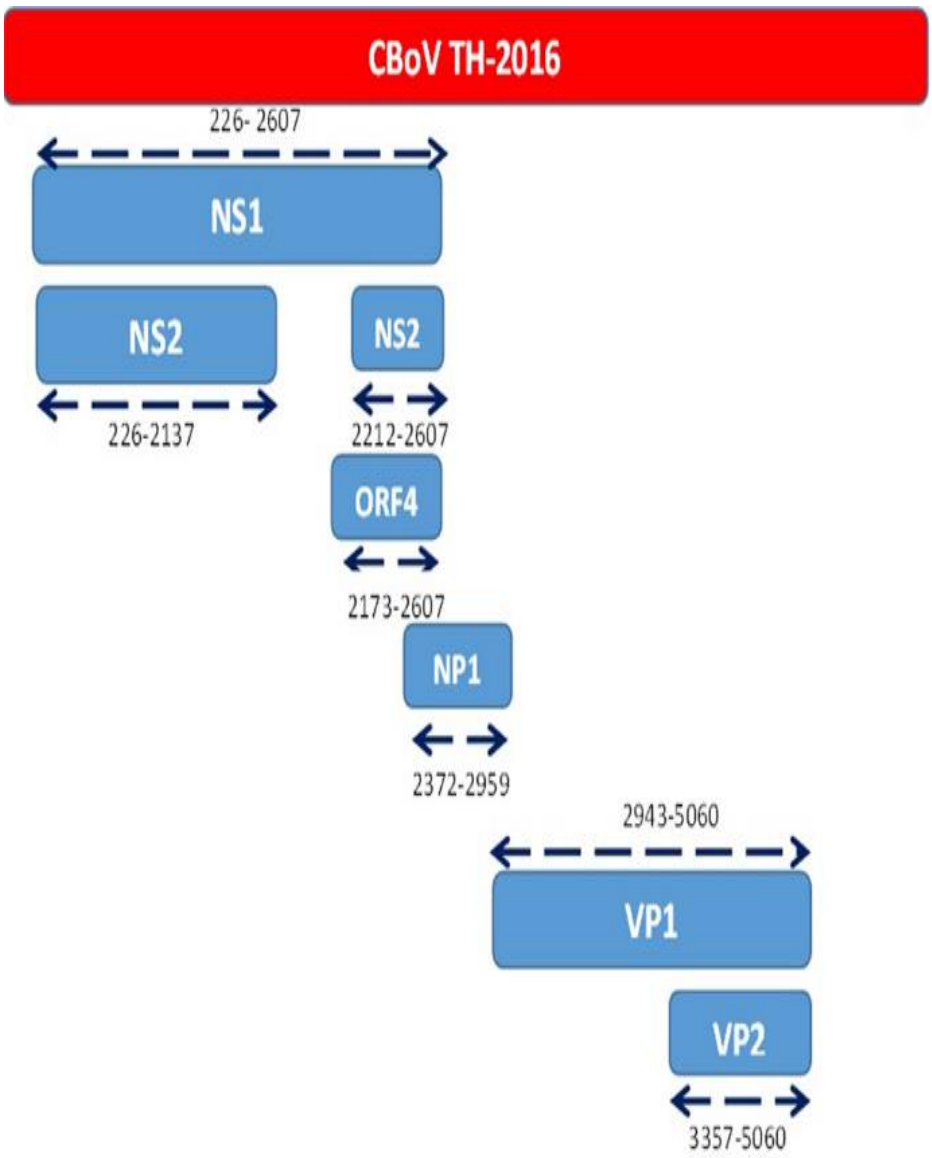

Figure 11. Genome organization of CBoV TH-2016 coding for NS1/2, NP1, ORF4, and VP1/2.

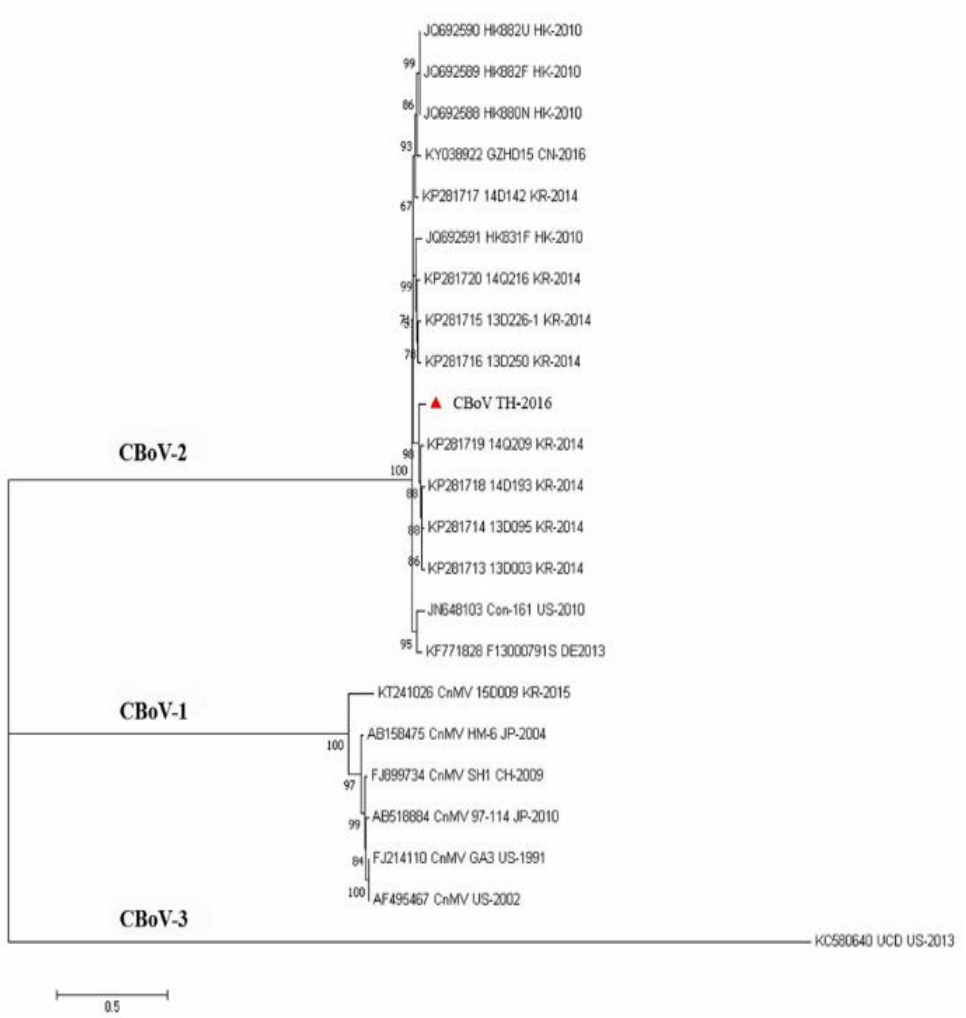

Figure 12. Phylogenetic tree of CBoV TH-2016 genome, constructed by maximum-likelihood algorithm with 1000 bootstrap replicates. CnMV: canine minute virus.

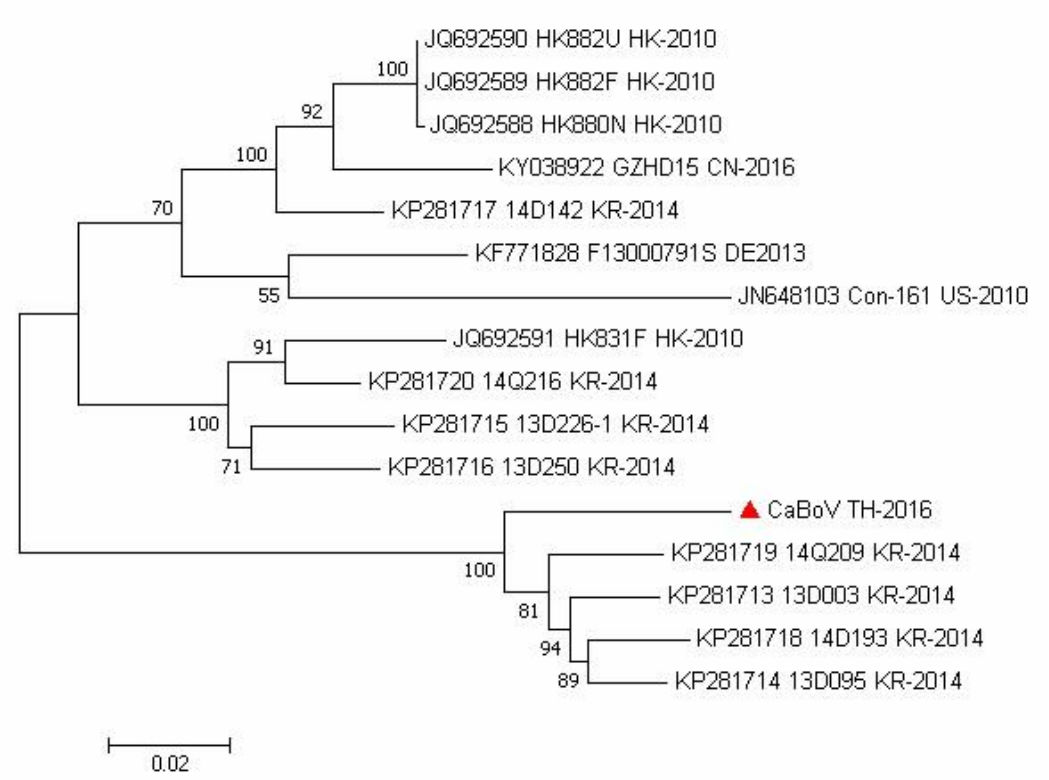

Figure 13. Phylogenetic tree of VP1/2 on CBoV TH-2016 genome, constructed by maximum-likelihood algorithm with 1000 bootstrap replicates.

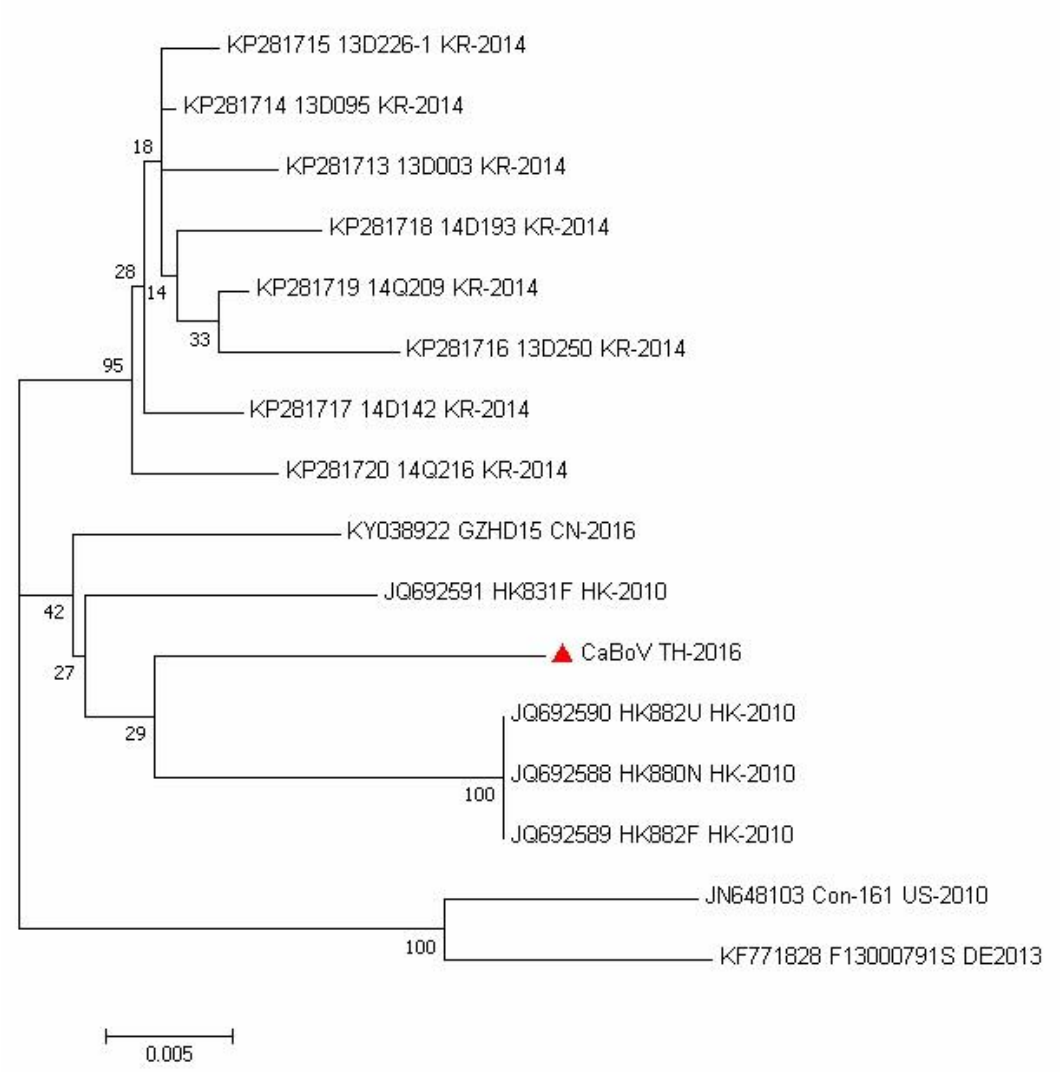

Figure 14. Phylogenetic tree of NS1 gene on CBoV TH-2016 genome, constructed by maximum-likelihood algorithm with 1000 bootstrap replicates.

Table 12. Pairwise comparison of nucleotide and deduced amino acid sequences of CBoVTH-2016 with other CBoV-2 strains in many countries.

CBoV-2 strains		Nucleotides/ Amino acids similarity (%)				
		Whole genome[a]	Non-structural regions		Structural region	ORF4[e]
			NS1/2[b]	NP[c]	VP1/2[d]	
USA, 2010	JN648103 Con-161	87.4/79.1	79.2/91.0	97.4/98.4	88.7/91.0	N/A
Hong Kong, 2010	JQ692591 HK831F	91.7/84.4	79.4/91.4	98.4/99.4	88.9/91.4	98.1/97.9
	JQ692590 HK882U	92.8/84.7	79.7/92.5	99.1/99.4	89.1/92.5	98.6/98.6
	JQ692588 HK880N	92.0/84.2	79.7/92.4	99.1/99.4	89.0/92.4	98.6/98.6
	JQ692589 HK882F	91.8/84.0	79.7/92.5	99.1/99.4	89.1/92.5	98.6/98.6
Germany, 2013	KF771828	91.2/82.8	80.7/91.7	96.7/98.4	89.0/91.7	N/A
South Korea, 2014	KP281720 14Q216	90.7/82.8	81.9/91.5	98.4/98.4	88.6/91.5	98.1/97.9
	KP281715 13D226-1	91.0/83.4	82.0/92.4	98.8/99.4	89.1/92.4	97.7/98.6
	KP281716 13D250	90.9/83.1	79.5/91.4	99.4/99.4	88.7/91.4	98.6/97.9
	KP281717 14D142	91.2/83.7	81.9/92.1	98.8/99.4	89.7/92.1	N/A
	KP281719 14Q209	93.2/87.6	81.4/97.5	98.8/99.4	94.7/97.5	97.9/97.9
	KP281718 14D193	93.3/87.8	97.7/97.8	99.3/99.4	94.8/97.8	97.9/98.6
	KP281714 13D095	93.2/87.5	97.2/98.1	98.8/98.9	95.1/98.1	N/A
	KP281713 13D003	91.6/86.0	82.0/97.5	98.9/99.4	95.2/97.5	N/A
China, 2016	KY038922 GZHD15	91.0/83.4	97.8/91.5	98.9/100	89.1/91.5	N/A

Abbreviation: N/A, no data available.

[a] 92.2/86.1% Mean similarity; [b] 91.9/94.4% Mean similarity; [c] 98.3/98.8% Mean similarity; [d] 90.1/94.4% Mean similarity; [e] 97.5/97.4% Mean similarity

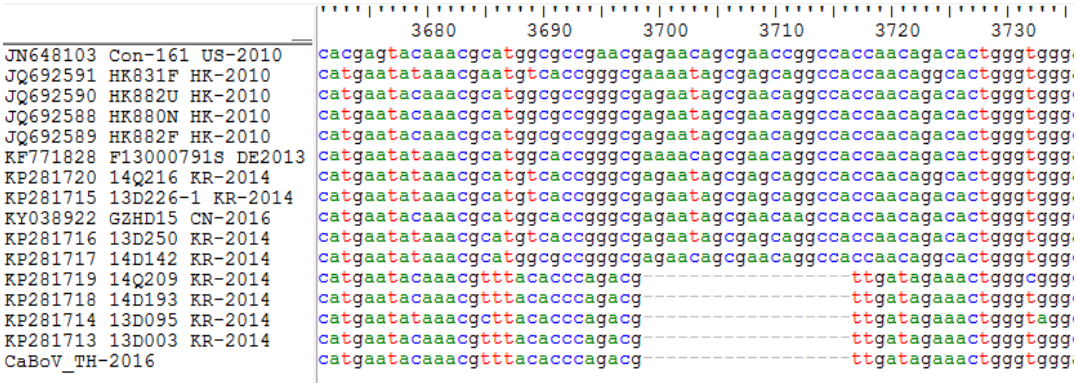

Figure 15. Nucleotide deletion in VP1/2 region. A deletion of 18 nucleotides is present in the strains 14Q209, 14D193, 13D095, 13D003 and TH-2016 of CBoV-2 between nucleotides 3,699-3,716, located at VP1/2.

Similarity plot and bootscan analysis revealed that CBoV TH-2016 shared sequences of putative parental CBoV-2 strains detected in South Korea and China-Hong Kong. A potential recombination break point was identified near the start codon of VP2 gene, which had high similarity to CBoV-2 detected in South Korea (Figs. 16-17).

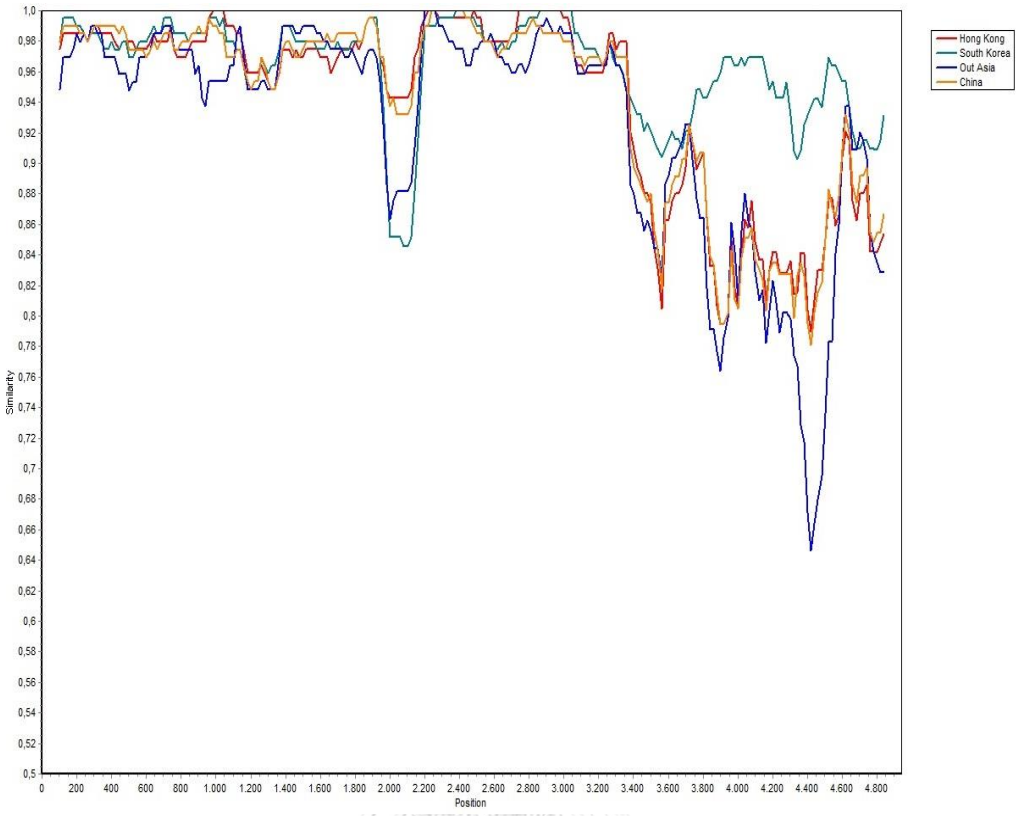

Figure 16. Similarity plot for the nearly complete genome sequence of CBoV TH-2016 strain. The CBoV-2 strain 13D095 (accession number: KP281714), HK882F (accession number: JQ692589 and GZHD15 (accession number: KY038922) were used as a represent strain of South Korea (green), Hong Kong (red), and China (yellow). The CBoV-2 strain Con-161_USA (accession number: JN648103) and F13000791S (accession number: KF771828) were used as Out Asia strain.

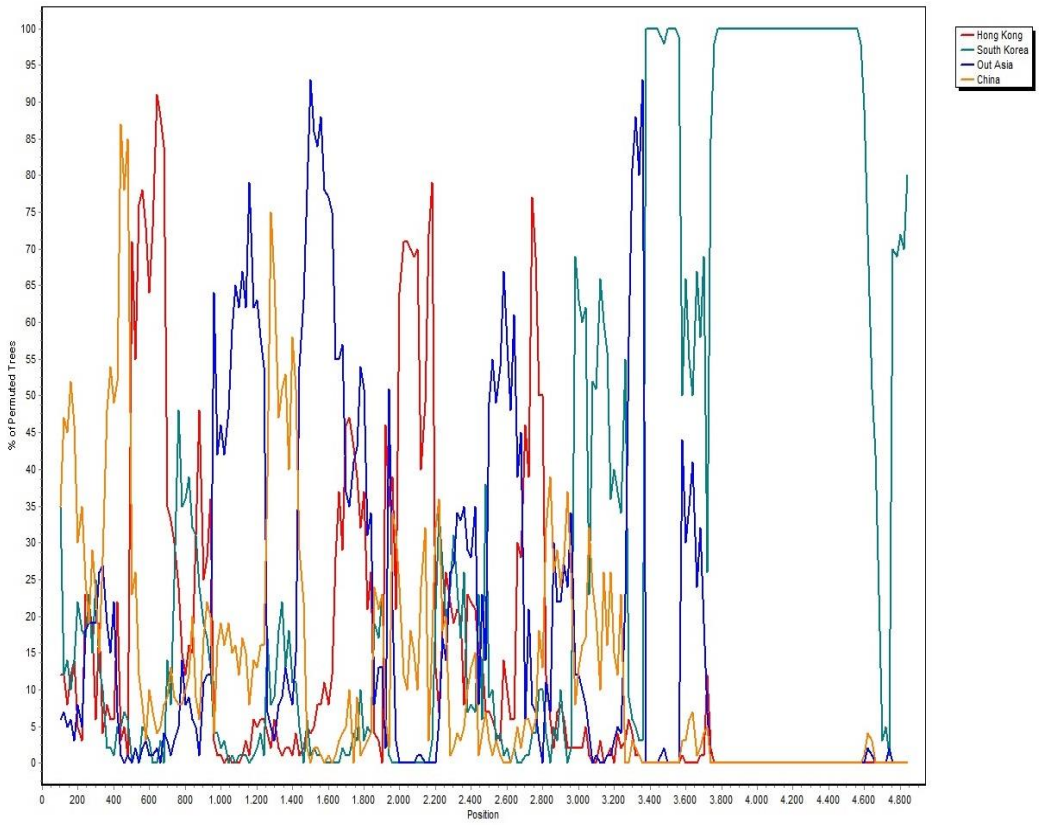

Figure 17. Bootscan analysis for the nearly complete genome sequence of CBoV TH-2016 strain. The CBoV-2 strain 13D095 (accession number: KP281714), HK882F (accession number: JQ692589 and GZHD15 (accession number: KY038922) were used as a represent strain of South Korea (green), Hong Kong (red), and China (yellow). The CBoV-2 strain Con-161_USA (accession number: JN648103) and F13000791S (accession number: KF771828) were used as Out Asia strain.

Screening tissues for the presence of CBoV-1 and CBoV-2

Due to the close relationship of CBoV-1 and CBoV2, the observation that intestinal lesions were found that had previously been considered pathognomonic for CBoV-1 infection, and confirmation of CBoV-2 infection in various organs in the absence of CBoV-1, CBoV-1 specific conventional PCRs were used to confirm the unique presence of CBoV-2, concordant with the NGS findings. The presence of CBoV-2 was identified in lung, liver, tracheobronchial lymph node and intestinal FFPE samples, whereas CBoV-1 could not be detected in any of the analyzed tissues. Sequences of all CBoV-2

positive samples were found to be identical to the NGS-derived CBoV-2 sequence. CBoV-2 was also detected by qPCR in all analyzed tissues except the lymph nodes of two dogs. The lowest Ct value was observed in FFPE-derived intestinal sample, followed by those of lung, liver and lymph nodes samples (Table 13).

Table 13. Detection of canine bocavirus-2 by using conventional PCR, quantitative PCR (qPCR) and in situ hybridization (ISH) in various organs of three respiratory suffering puppies.

Animal	Samples	Methods		
		PCR	qPCR (Ct value)	ISH
Case No. 1	Lung	+	+ (20.00)	-
	Intestine	+	+ (13.72)	+
	Liver	+	+ (26.71)	-
	Lymph node	-	+ (30.44)	-
	Others[a]	N/A	N/A	-
Case No. 2	Lung	+	+ (20.17)	-
	Intestine	+	+ (15.00)	+
	Liver	+	+ (20.23)	-
	Lymph node	+	-	-
	Others[a]	N/A	N/A	-
Case No. 3	Lung	+	+ (20.4)	-
	Intestine	+	+ (19.24)	+
	Liver	+	+ (15.74)	-
	Lymph node	+	-	-
	Others[a]	N/A	N/A	-

Abbreviation: N/A, no data available.

[a] Other organs including gall bladder, heart, kidneys, pancreas, spleen and trachea were tested with ISH.

In situ hybridization and transmission electron microscopy

Tissue samples from the three pups, including heart, trachea, lung, lymph nodes, liver, gall bladder, pancreas, small intestine, kidneys and spleen were examined by ISH to determine the cellular tropism of CBoV-2. Many small intestinal enterocytes in variable locations displayed a nuclear signal for CBoV-2 (Fig. 18). Similarly, a positive signal was obtained in sloughed enterocytes within the small intestinal lumen. No ISH signal was detected in any of the other tissues investigated. Within several cross sections of the small intestine, a nuclear signal for CBoV-2 was observed in many enterocytes, which were mainly located at the villus tips (Fig. 18) and to a lesser extend within crypts. Similarly, a positive signal was obtained in sloughed enterocytes within the small intestinal lumen, compared with the negative control sample (Fig. 19).

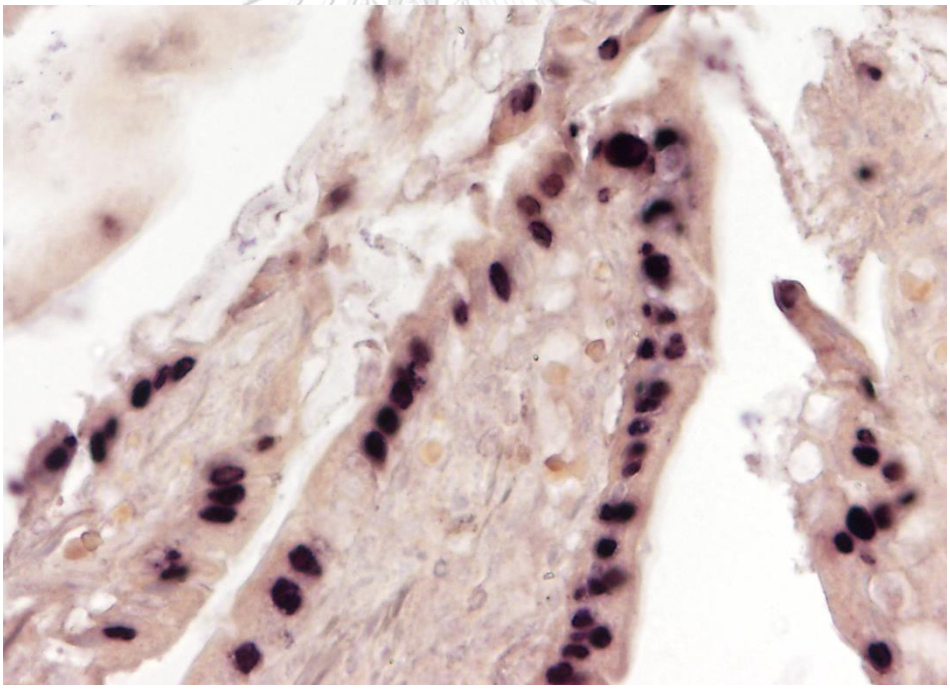

Figure 18. Canine bocavirus-2 infection, small intestine, dog. Strong positive signals (brown color) within the nuclei of enterocytes corresponding to the INIB seen in HE. In situ hybridization (ISH).

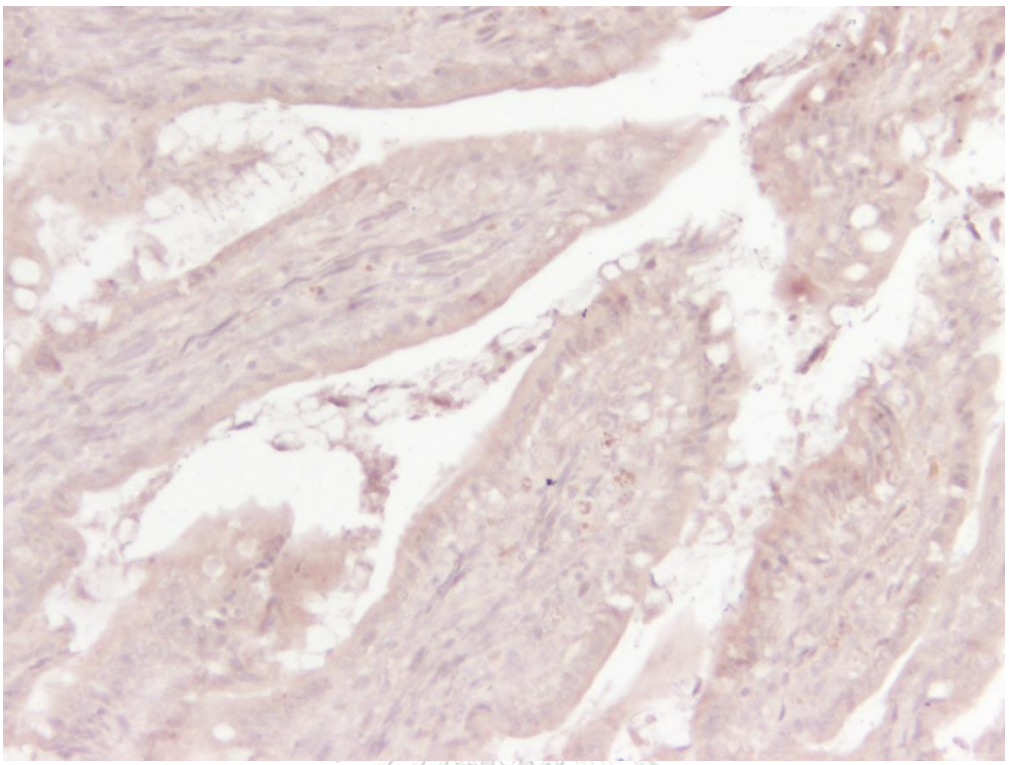

Figure 19. Canine bocavirus-2 infection, small intestine, dog. No reaction is present within a non-probe control. In situ hybridization (ISH).

Transmission electron microscopy was used to verify the presence of virus particles within apical small intestinal enterocytes. Numerous electron dense, spherical virus particles, measuring about 20 nm in diameter were found, aggregated to large, intranuclear inclusion bodies in these cells (Fig. 20).

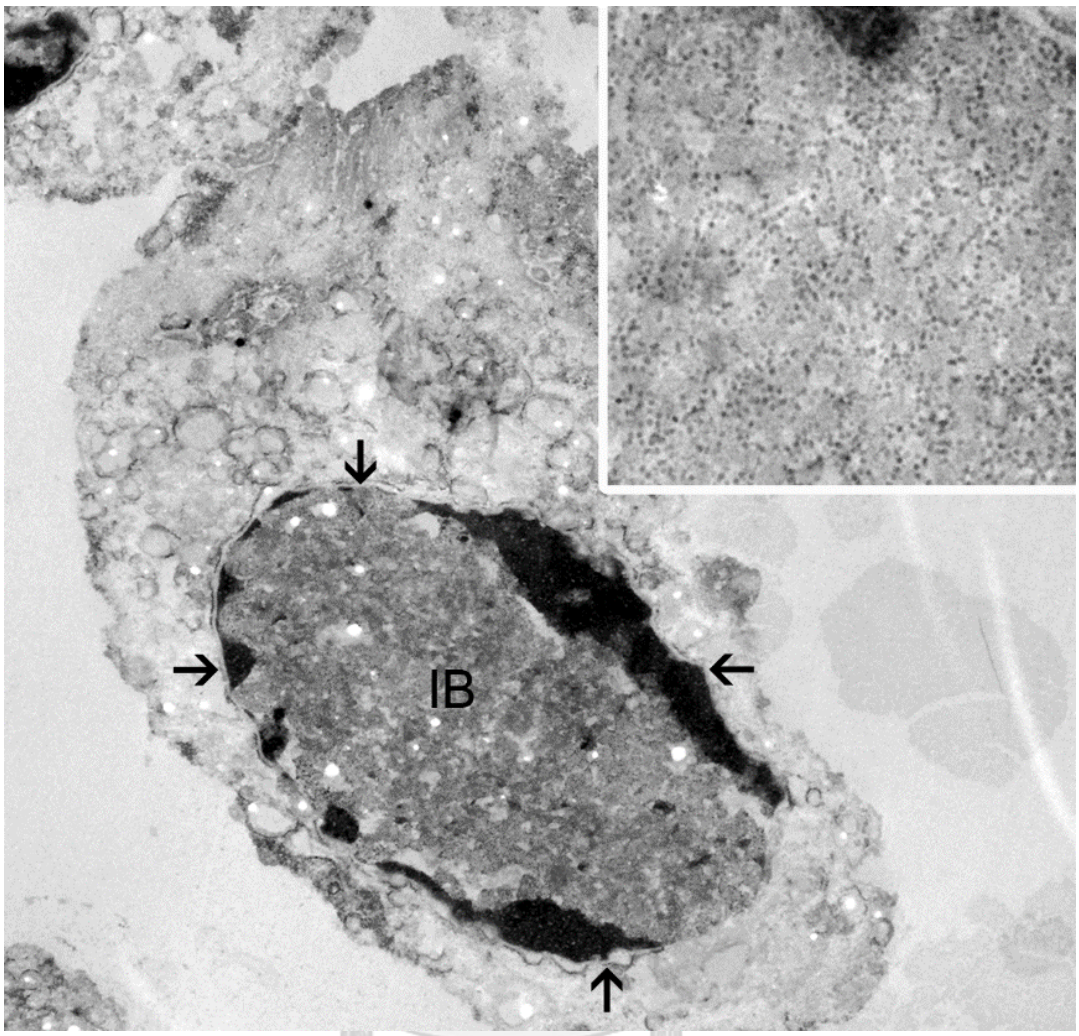

Figure 20. Canine bocavirus-2 infection, small intestine, dog. An intranuclear inclusion body (IB) is present within the nucleus (arrows) of a small intestinal enterocyte characterized by aggregates of numerous virions (inset). Transmission electron microscopy.

DISCUSSION

In the present study, we have investigated the etiology of an unknown disease associated with the deaths of a litter of pups in Thailand. Although respiratory failure was initially suspected, gross- and histopathological analyses did not show any pathology in respiratory tissues. Routine diagnostic post-mortem investigations aiming at identifying pathogens known to be associated with canine respiratory disease were also negative. Consequently, the cause of death could not be determined. This prompted us to search for other viral pathogens by NGS, resulting in the identification of CBoV-2 in all pups. Recently discovered viruses of the genus *Bocaparvovirus* have been associated with gastrointestinal and respiratory disease in several mammalian species, including humans and dogs (Schildgen et al., 2008; Bodewes et al., 2014; Choi et al., 2015; Guido et al., 2016). Based on comparative phylogenetic analyses of the nucleotide sequences generated, CBoV TH-2016 was found to be a novel strain of CBoV-2, most closely related to previously identified CBoV-2 strains from South Korea and Hong Kong. However, the histopathological presentation of CBoVTH-2016 infection was different from that of previously described CBoV-2 infections (Bodewes et al., 2014; Choi et al., 2015), and rather similar to that described for natural and experimental CBoV-1 (or CnMV) infections: intranuclear inclusion bodies in enterocytes without substantial inflammatory reaction (Carmichael et al., 1994; Jarplid et al., 1996). In contrast, the enteric presentation of previously identified CBoV-2 infections in pups was absent: no severe enteritis with massive atrophy of villi and bone marrow and generalized lymphoid depletion, with evidence of virus replication in the cytoplasm of lymphoid cells were found (Bodewes et al., 2014). Most of the viruses belonging to the *Parvoviridae* family replicate in cells with a high mitotic activity such as intestinal crypt epithelial cells; however, recent studies demonstrated the presence of parvoviruses in typically non-mitotic cells such as neurons (Schaudien et al., 2010; Garigliany et al., 2016; Pfankuche et al., 2017). Furthermore, the pathogenesis of bocaviruses regarding tissue tropism and replication is not well documented. Close genetic relationship to CBoV-1, which shows viral inclusion bodies in enterocytes (Harrison et al., 1992), may

serve as a possible reason. Therefore, further studies of CBoV-2 pathogenesis should be conducted.

The demonstration of CBoV-2 in lungs and other organs by NGS and PCR, in the absence of CBoV-2 ISH signal outside the intestinal tract was indicative of hematogeneous spread of CBoV TH-2016 from the infected intestinal tract. Similar findings of PCR positive but ISH negative lungs in CBoV-2 infected dogs were also recently reported (Choi et al., 2015), and are consistent with previous detections of CBoV-2 in both respiratory- and intestinal tract samples (Kapoor et al., 2012b; Lau et al., 2012; Bodewes et al., 2014; Choi et al., 2015). Since CBoV-1 has been associated with enteric lesions quite similar to those described here (Binn et al., 1970), we confirmed absence of CBoV-1 infection in these pups using four different CBoV-1 specific PCR assays carried out on the tissues that were CBoV-2 positive.

The newly identified CBoV TH-2016 was found to contain a unique 18 nucleotide deletion in the VP2 gene of CBoV-2, a finding previously reported to be associated with respiratory disease in pups (Kapoor et al., 2012b), while CBoV-2 strain F13000791S, associated with severe enteritis, did not contain this deletion (Bodewes et al., 2014). However, the significance of this deletion for association with respiratory disease remains to be determined, as no amino acid deletions were detected in the VP2 gene in another recent case of a CBoV-2 infection of a dog with severe respiratory disease (Choi et al., 2015). Interestingly, the presence of ORF4 in CBoV TH-2016, which was not associated with any pathology in respiratory tissues, is in line with the observation of absence of ORF4 in dogs with respiratory disease (Choi et al., 2015). Finally, CBoV TH-2016 has a relatively long NS1 region, encoding 793 amino acids. This finding is consistent with a previous study suggesting that some strains of CBoV-2 genome possess a putative second exon encoding the C-terminal region of NS1 and conserved RNA-splicing signals close to the NS1 gene that may lead to the generation of a longer NS1 protein (Kapoor et al., 2012b; Lau et al., 2012). Recent studies have documented however that the longer NS1 protein is observed in dogs showing respiratory problems (Kapoor et al., 2012b; Choi et al., 2015).

In contrast to many DNA viruses which show limited evolutionary kinetics, parvoviruses are capable of rapid evolution resulting in novel species and genotypes

and species with similar evolutionary dynamics to that observe for RNA viruses (Nguyen et al., 2002; Servant et al., 2002; Duffy et al., 2008). Similarly, recent studies have indicated that human and animal bocaviruses, such as HBoV, porcine bocavirus (PBoV) and feline bocavirus (FBoV), readily undergo genetic rearrangement and recombination (Hogan and Faust, 1986; Hoelzer et al., 2008; Kapoor et al., 2009; Kapoor et al., 2010; Lau et al., 2011; Lau et al., 2012). Phylogenetic analyses of the CBoV-TH-2016 genome and VP1/2 sequences show a close evolutionary relationship with CBoV-2 strains detected in South Korea. However, analysis of the NS1 region alone suggested that this strain is most closely related CBoV-2 strains identified in Hong Kong. Indications for genetic recombination located at VP1/2 gene of CBoV TH-2016 as identified in this study should be subject to further study.

Since these studies collectively indicated that a unique deletion in VP1/2 gene, the absence of ORF4, and the presence of a longer NS1 gene may all be associated with changes in viral pathogenesis and virulence of CBoV-2 (Kapoor et al., 2012b; Choi et al., 2015), further studies linking viral pathogenesis and virulence with molecular CBoV-2 markers are warranted. As clinical manifestations and genetic organization of animal and human bocaviruses appear to be similar, further studies of natural and experimental infections of bocaviruses in dogs may shed light on the molecular basis of the pathogenesis and virulence of members of the genus *Bocaparvovirus* in humans and animals.

Taken together, we have identified a novel strain of CBoV-2 (CBoV TH-2016) that shares genetic traits with previously identified strains in Hong Kong and South Korea, with evidence for genetic recombination. Naturally infected dogs showed histopathological intestinal lesions reminiscent of those described in CBoV-1 infection, and quite distinct from those hitherto described for CBoV-2 infections. Using NGS and specific tests for the novel virus should be taken into account for clinical dogs showing negative results in a routine laboratory diagnosis as an emergence of novel recombinant CBoV-2 may lead to atypical syndrome. Although the cause of death of the pups could not be established unequivocally, a contributory role of enteric CBoV TH-2016 infection cannot be excluded. Using NGS and specific tests for the novel virus should be taken into account for diseased dogs showing negative results in routine

laboratory investigations as an emergence of novel recombinant CBoV-2 may lead to atypical findings. Although the cause of death of the pups could not be established unequivocally, a contributory role of enteric CBoV TH-2016 infection cannot be excluded.

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2.5 GENOMIC ANALYSIS OF NOVEL CANINE CIRCOVIRUS STRAINS FROM THAILAND: EVIDENCE FOR NATURAL GENETIC RECOMBINATION

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ABSTRACT

Circoviruses are non-enveloped, circular, single stranded DNA viruses contain 2kb genome length and belonging to *Circoviridae* family, which is either primary or secondary cause of systemic diseases in many animals. Recently, canine circovirus (CanineCV) was discovered and believed that it was associated with enteric and systemic vasculitis and hemorrhagic diseases. In the present study, we detected the CanineCV from acute respiratory suffering dogs located in Thailand. Genome characterization of detected CanineCVs in Thailand suggested there were two novel CanineCV strains circulating in Thailand and were distinct from other published strains. Natural recombination event among different CanineCV lineages of USA and China was carried out by using similarity plot and bootscanning. The recombination breakpoint was located in origin of replication (Ori) and rep gene with statistical significance using cocktails of maximum-likelihood framework. Interestingly, novel ORF3 was discovered in these strains. Conserved DNA detection and *in situ* hybridization were performed in several organs, suggesting tissue distribution of CanineCV. Here, we established the naturally-novel recombinant CanineCV strains as a first report. This study emphasized the recombination among CanineCV strain may contribute the novelty and might be a possible cause of respiratory problem in dogs.

Keywords: Canine circovirus, *Circoviridae*, CIRD, Genetic, Recombination

INTRODUCTION

Circoviruses are non-enveloped, circular, single stranded DNA viruses contain 2kb genome length (Li et al., 2013a). The viruses belong to family *Circoviridae*, genus *Circovirus*, which contained both 2 types of porcine circovirus (PCV1 and 2) affecting pigs, canary circovirus (CaCV), beak and feather disease virus (BFDV) affecting both domestic and wild birds (Tischer et al., 1986; Todd et al., 2001; Todd, 2004; Hsu et al., 2016). In 2012 during massive metagenomics investigations, a novel mammalian circovirus namely canine circovirus-1 (CanineCV-1), was detected from dog sera, representing a circovirus apart from porcine circoviruses (Kapoor et al., 2012a). The circovirus in dogs was initially named as DogCV; however, the name was now established by the International Committee on Taxonomy of Viruses (ICVT) as "CanineCV" (Anderson et al., 2017). The CanineCV was initially documented that it was associated with vasculitis and hemorrhage and also it was discovered from blood stream of dogs showing thrombocytopenia and neutropenia with unknown origin (Li et al., 2013a). Later on, several studies investigated the CanineCV in fecal samples and suggested that this virus may serve as a possible pathogen causing diarrhea (Li et al., 2013a; Decaro et al., 2014; Hsu et al., 2016; Anderson et al., 2017); however, the CanineCV could be also detected in healthy dogs (Li et al., 2013a). Interestingly, the CanineCV often co-infected with other enteric or respiratory pathogens such as canine parvovirus (CPV), *Giardia sp*., canine enteric coronavirus (CCoV) and canine distemper virus (CDV) (Hsu et al., 2016; Thaiwong et al., 2016; Zaccaria et al., 2016; Anderson et al., 2017). Recent studies, therefore, attempted to investigate the correlation of enteric-associated diseases with the CanineCV infection such as severities of diarrhea, age of affecting dogs, possible chance of co-infection and pathological appearance. Despite clinical severities of diarrhea were did not significantly associate with the infection (Hsu et al., 2016), the clinical signs lead to significantly higher mortality rates whenever co-infection with the CPV (Anderson et al., 2017). The role of CanineCV associate with acute hemorrhagic diarrhea syndromes (AHDS) was also studied; however, no association between the virus and the syndromes was documented (Anderson et al., 2017).

Most of CanineCV infected dogs showed lymphadenopathy and hemorrhage, which was frequently observed in gastrointestinal tract, followed by multiple dissemination in kidneys, liver, spleen, lung and many parts of brain and meninges (Li et al., 2013a). Multiple necroses of lymph nodes and vessels were seen in Payer's patches and corticomedullary junction and tubules in kidneys; however, multinucleated giant cells, but not epithelioid macrophages, were not commonly observed as frequently seen in the PCV-2 infection (Thaiwong et al., 2016). Distribution of viral nucleic acids in various tissues was detected by *in situ* hybridization, suggesting that the CanineCV only contained in cytoplasm of macrophages in various lymphoid organs as its cellular tropism; even though the CanineCV genome was detected in various organs by using PCR (Li et al., 2013a). Nonetheless, granulomatous inflammation, a hallmark of PCV-2 infection in pig, was presented with a few ISH-specific CanineCV located in nuclei of regenerating cryptal epithelial cells of small intestine and Kupffer cells in liver (Thaiwong et al., 2016).

Genetic mutations and recombination are major effects for viral evolution. Several evidences of genetic recombination occurring in viruses belonged to *Circoviridae* were reported during last decade. Genetic recombination has been reported in naturally infected animals with Beak and Feather disease virus (Heath et al., 2004) and Torque teno virus (Manni et al., 2002). Moreover, even analysis of PCV-2 genome distributing worldwide revealed more than 90% genetic identity among the PCV-2a and -2b (Hamel et al., 1998; Fenaux et al., 2000); recent studies documented the evidences of genetic recombination naturally occurring among PCV-2 strains (Csagola et al., 2006; Ma et al., 2007; Olvera et al., 2007; Hesse et al., 2008). The evidence of genetic recombination in the various *Circoviridae* viruses might, therefore, have contributed to genetic variation and shed the possibility of genetic recombination in other viruses belong to this family. Since distribution of the CanineCV-infected dogs was documented in many countries such as USA (Kapoor et al., 2012a; Li et al., 2013a; Thaiwong et al., 2016), Italy (Decaro et al., 2014), Germany (Anderson et al., 2017), China (GeneBank accession number: KT946839) and Taiwan (Hsu et al., 2016) with intensive reports of its several strains, neither genetic recombination nor mutation of CanineCV genome were evident. In this study, we aimed to characterize viral genome

and establish an evidence of genetic recombination of CanineCV, along with the pathological finding of the naturally-infected dogs circulating in Thailand

MATERIALS AND METHODS

2.1 Animal description and sample collection

Three dog tissues (14P105D, 14P112N, 15P061D) were collected from routine post mortem examination at Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. Several organs were preserved in 10% neutral formalin for further histopathology examination. Fresh tissues consisting of brain, tonsil, lung, tracheobronchial lymph nodes, liver, and kidneys were collected at -80^{O}C for molecular assays.

Moreover, twenty nasal or oral swabs sampling from Thai dogs that presented various degrees of respiratory problems and ten necropsied dog's lungs showing various lung lesions from Department of Pathology, University of Veterinary Medicine, Hannover, Germany were also subjected in this study.

2.2 Sample preparation and cDNA synthesis

All fresh tissue and the swab samples collecting from Thailand were subjected to routine virological diagnosis. The fresh tissues were individually homogenized under aseptic conditions. Total viral nucleic acids were extracted using Viral Nucleic Acid Extraction Kit II (GeneAid, Taipei, Taiwan) according to manufacturer's recommendation. The nucleic acid was quantified and qualified using Nanodrop® Lite (Thermo Fisher Scientific Inc., Waltham, MA, USA). Total cDNA was synthesized by using 100 *n*g RNA as the template for complementary DNA (cDNA) synthesis using the Omniscript® Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany). The cDNAs were stored at −20°C until used for further PCR amplification.

2.3 Routine laboratory testing

All cDNAs were further subjected for routine laboratory investigations. Common viruses of canine infectious respiratory disease complex (CIRDC) including canine

influenza virus (CIV), canine parainfluenza virus (CPIV), canine distemper virus, (CDV), canine respiratory corona virus (CRCoV), canine adenovirus type 1 and 2 (CAdV1-2) and canine herpesvirus type 1 (CaHV-1) were screened by using mPCRs (Piewbang et al., 2016) Moreover, the pan-PCRs specific for paramyxovirus, coronavirus and herpesvirus were done on the extracted nucleic acid as described previously (VanDevanter et al., 1996; Ksiazek et al., 2003; Tong et al., 2008) with some modifications. Available dual commercial kit, Rapid CPV/CCV Ag test kit (Bionote Inc., Suwon, South Korea), was used in three dogs during necropsy, ruling out the canine parvovirus (CPV) and canine enteric coronavirus (CCV) infections.

2.4 Sequence independent single primer amplification and next-generation sequencing.

Because of most dogs showing respiratory problems, lung samples of dogs No.14P105D and No. 14P112N were prepared for deep sequencing using a modified sequence-independent single-primer amplification (SISPA) protocol. Briefly, after tissue homogenization, RNA was extracted by Qiamp Viral RNA mini kit (Qiagen, Hilden, Germany) following manufacturer's recommendation. cDNA was constructed from extracted RNA by using a mixture of random and non-ribosomal hexamers (Allander et al., 2001; Endoh et al., 2005) with SuperScript IV (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), followed by Klenow reaction. Subsequently, Taq polymerase (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was used to randomly amplify the cDNA. The PCR product was then purified by Monarch® PCR and DNA clean up kit (New England Biolab, Frankfurt, Germany). Final DNA concentration was measured by PicoGreen (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). A DNA Library was subsequently constructed following Nextera XT protocol (Illumina, USA). Samples were then deep sequenced on an Illumina MiSeq system using MiSeq Reagent kit V3 (300x2 cycles).

Raw reads were initially screened by an *in house* metagenomics pipeline to identify interesting viral reads. Reference assembly was performed using CLC Genomics Workbench 9.0. Phylogenetic analyses were carried out using MEGA 7.

2.5 Genome sequencing and CanineCV-specific PCR

PCR with Sanger sequencing were performed to confirm a presence of interesting viral genome. The various CanineCV strains were retrieved from GenBank database and were aligned with the NGS-derived reads from this study, namely CanineCV_14P105D/TH2014 and CanineCV_14P112N/TH2014 by using BioEdit (Ibis Biosciences, Carlsbad, CA, USA). Nearly complete CanineCV genomes were obtained by PCR amplification using degenerated primers; all were designed from various strains of CanineCV (Table 14.). Briefly, PCR reactions comprised of a mixture of 1U of Phusion DNA polymerase (New England Biolab, UK), 10 mM of dNTP in 5x Phusion HF Buffer, 10 *μ*M final concentration of each primer and 2 *μl* of template. Cycler conditions consisted of initial denaturation for 98 ºC for 30 sec, followed by 45 cycles of 98 ºC, 20 sec, 50 ºC, 30 sec, 72 ºC, 1 min and 72 ºC, 7 min for final extension. The PCR product was run in 1% agarose gel electrophoresis and purified by using Monarch DNA Gel extraction kit (New England Biolab, Frankfurt, Germany). All purified amplicons were submitted to Sanger sequencing in order to determine the complete nucleotide sequences of detected CanineCV. The nearly complete genomes of CanineCV were deposited in GenBank.

2.6 Canine Circovirus in other animals and respiratory swab samples

The genetic diversity of CanineCV strains was investigated in lungs of dog No. 15P061D, ten lung samples and 20 respiratory swabs, derived from dogs showing various respiratory problems, by specific CanineCV PCR amplification and sequencing. Primers were designed from various CanineCV strains available in GenBank, targeting 517 bp of partial capsid gene (Table 14.). Later, a panel of primers for amplification of nearly complete genome of CanineCV was performed in the samples that were positive from the CanineCV-PCR screening as mentioned above. The composition and condition of PCR assay were as same as described in 2.5. After electrophoresis, amplicons were purified and sent for sequencing.

Table 14. Sequences of CanineCV specific primers used for complete genome characterization.

Genome position	Sequence (5'-3')	Direction	Product size (bp)
121-140	GCGCCGGACGCTAAGTACTT	Forward	968
1067-1088	GTCACGWGTGTTTATTRGYYGG	Reverse	
1022-1044	TTTACCTGTTCACCCCCCTTCGA‡	Forward	517
1515-1538	GGAAGAGGYAATGCTACAAGATCA‡	Reverse	
1306-1324	GTKCCTCTKGTYAGCCATG	Forward	741
2028-2046	GTGCTGTGTCTGTGACGAG	Reverse	
605-626	AATGGTGGGAYGGYTACGATGG*	Forward	437
1021-1041	AAGGGGGGTGAACAGGTAAAC*	Reverse	

‡Primers used for complete CanineCV whole genome and also used as primers for CanineCV-specific PCR targeting capsid gene region.

* Primers used for filling the gap of CanineCV genome, which achieved from NGS read.

2.7 Sequence and genomic recombination analysis

The CanineCV sequences of all positive samples were re-multiple aligned using MAFFT alignment version 7 (http://mafft.cbrc.jp/alignment/server/) and MEGA 7 based on nucleotide sequences and deduced amino acids of CanineCV genome. Phylogenetic trees were constructed based on nearly complete genome, replicase (Rep) and capsid (Cap) genes of represent CanineCV strains in GenBank by using neighbor-joining method with TN93+G for complete genome, K2+G model for Rep gene and HKY+G for Cap

gene. Maximum likelihood and bootstrap analysis was performed using 1000 replicates. Sequence pairwise distances of CanineCV genomes were calculated using Maximum Composite Likelihood model (Tamura et al., 2004). The evolutionary analyses were conducted in MEGA 7 (Kumar et al., 2016).

All Thai isolated CanineCV sequences were tested for genomic recombination by using recombination detection program 4 (RDP4) package v. Beta 4.94 (Martin et al., 2015). Briefly, those CanineCV strains were integrated to compute by the RDP4 for further investigation an evidence of possible recombination region. Different recombination assays including RDP, GeneConv, Bootscan, MaxChi, Chimera, SiScan and 3Seq were accessed for those sequenced CanineCV strains with statistically acceptable at *p*-value less than 0.01. Bonferroni correction was used as a general setting. The other complete genomes of CanineCVs strains available in GenBank were included in the RDP analyses.

Furthermore, similarity plot and bootscan in SIMPLOT software package version 3.5.1 were used for recombinant analysis (Lole et al., 1999). Briefly, a bootscaning analysis was performed on various groups of complete CanineCV genome sequences based on different clusters of phylogenetic tree. The groups were consisted of CanineCV strain UCD3-478 (accession no. JQ821392), CanineCV-US (accession nos. KC241982, KC241984, JQ821392), CanineCV-IT (accession nos. KJ530972, KT734812-KT734828, KJ530972, KC241984), CanineCV-DE (accession nos. KF887979, KT283064) and CanineCV-CN (accession no. KT946839). Potential recombinants Thai CanineCV strain (CanineCV_14P105D/TH2014, 15P112N/TH2015, CP28/TH2014, CP134/TH2015, CP144/TH2015, CP182/TH2014, CP188TH/2014) were used as a query. The bootscan analysis was tested under the Kimura-2 parameter (K2P) model for 100 replications, with a window size of 200 bp, step size of 20 bp and a transition/transversion ratio of 2. The similarity plot was performed using the same groups and conditions with GapStrip model. An identified recombinant sequences was also allegorized with their potential parents by genetic marker positions in order to achieve further evidence of genetic recombination.

2.8 CanineCV genome distribution and in situ hybridization

When CanineCV was positive by PCR in lungs of necropsied dogs, other remaining fresh tissues of each animal, including brain, liver, tracheobronchial lymph node, tonsil and kidneys, were assayed to detect the CanineCV genome by using primers targeting the capsid gene (Table 14.). After cDNA synthesis, PCR reactions consisting 5x OneTaq Buffer, 10 mM of dNTP, 1U Hot start Taq polymerase (New England Biolab, Ipswich, MA, U.S.A.), 10 *μ*M final concentration of each primer and 2 *μl* of cDNA, were done. Cycler conditions contained initial denaturation for 94 ºC for 5 min, followed by 45 cycles of 94 ºC, 30 sec, 50 ºC, 30 sec, 72 ºC, 1 min and 72 ºC, 7 min for final extension. After electrophoresis, the CanineCV positive PCR products were purified prior submitting for sequencing.

To confirm the presence of CanineCV, *in situ*-hybridization (ISH) was performed on FFPE tissues, including lymph nodes, tonsils, thymus, spleen, pancreas, small intestine, kidney, urinary bladder, heart, trachea and lungs. ISH was performed as previously described with minor variations (Bodewes et al., 2014). Probes labelled with Digoxigenin (DIG) that covered 25 bp of the capsid gene of the aligned canine CV (accession nos: KC241982-84, JQ821392, KJ530972, KT734812-28, KJ530972, KC241984, KF887979, KT283064 and KT946839; nt 905-929) were used. Briefly, after de-paraffinization of FFPE sections and hydration in graded ethanol, samples were washed in diethyl pyrocarbonate (DEPC)-treated water. Proteolytic digestion of samples was performed with 1 µg/ml proteinase K (Roche Diagnostics, Basel, Switzerland). An overnight incubation at 52°C with 1000 ng/100 µl sense and antisense probe, respectively, was performed following post-fixation, acetylation and pre-hybridization. Detection was carried out using an anti-DIG antibody (diluted 1:200) conjugated with alkaline phosphatase (Roche Diagnostics, Basel, Switzerland) in combination with nitrobluetetrazoliumchloride (NBT, Sigma-Aldrich, St. Louis, MO, U.S.A.) and 5-bromo-4-chloro-3-indolyl phosphatate (BCIP, X-Phosphate, Sigma-Aldrich, St. Louis, MO, U.S.A.) as substrates. All distinct, purple precipitates, that were clearly cellular located, were considered positive.

RESULTS

3.1 Post mortem examination

Gross and histological examinations in all three dogs revealed various degrees of pneumonia. Most of them showed severe reddish and gray color of caudal and cranial lung lobes, respectively. Moderate multifocal hemorrhage with alveolar emphysema and moderate multifocal suppurative bronchopneumonia along with multifocal alveolar histiocytosis were noted in dog no. 14P105D. Severe diffuse suppurative fibrinous (dog no. 14P112N) and necrosuppurative (dog no. 15P061D) bronchopneumonia were presented. Mild lymphoid hyperplasia of tracheobronchial lymph node and tonsil were examined in dog no. 14P105D and 14P112N, whereas moderate sinus histiocytosis and plasmacytosis with single multinucleated giant cells were shown in dog no. 15P061D. Moreover, evidence of fungal organism, differentially Cryptococosis, was seen in lung of dog no. 15P061D. Moderate periportal lympho-histiocytic hepatitis and moderate multifocal lympho-histiocytic vasculitis were presented in dog no. 15P061D (Table 15.).

3.2 Routine laboratory investigation

A diagnostic panel of common canine respiratory viruses targeting CDV, CIV, CPIV, CRCoV, CaHV-1, CAdV-1 and 2, demonstrated the PCR positive CIV and CRCoV in lung of dog no. 14P015D and the positive CPIV in dog no.14P112N. No any viruses were detected in dog no. 15P061D and all 20 respiratory swabs by using the mPCR, the pan PCR for coronavirus, paramyxovirus and herpesvirus (Table 15.). Neither CPV nor CCoV were detected in intestinal swab of those 3 autopsied dogs by using dual antigen test kit.

Table 15. Animal history, significant clinical or pathological findings, sample tested and GenBank accession number of CanineCV genomes sequenced.

Sample No.	Year	Age	Sex	Clinical or pathological findings	Samples	CanineCV investigation			Other pathogens detected
						NGS	PCR	ISH	
14P105D	2014	7 m	Male	● Lymph node, tonsil: Mild lymphoid hyperplasia and mild to moderate sinus histiocytosis of the lymph node ● Focal lympho-histiocytic interstitial nephritis ● Severe diffuse suppurative bronchopneumonia	Lung	+	+	-	CIV, CRCoV
					Liver	N/A	+	-	
					Kidneys	N/A	NSA	-	
					Brain	N/A	-	N/A	
					Tonsil	N/A	+	+	
					LN	N/A	+	+	
					Others*	N/A	N/A	-	
14P112N	2014	1 m	Female	● Tonsil: Mild, lymphoid hyperplasia ● Severe diffuse suppurative bronchopneumonia	Lung	+	+	-	CPIV
					Liver	N/A	+	-	
					Kidney	N/A	NSA	-	
					Brain	N/A	-	N/A	
					Tonsil	N/A	+	-	
					LN	N/A	+	-	
					Others*	N/A	N/A	-	
15P061D	2015	4 y	Female	● Lymph node: Moderate sinus histiocytosis and plasmocytosis; single multinucleated cells ● Mild to moderate, lympho-histiocytic,	Lung	N/A	+	-	-
					Liver	N/A	+	-	
					Kidney	N/A	+	N/A	
					Brain	N/A	-	N/A	
					Tonsil	N/A	NSA	N/A	
					LN	N/A	+	-	
					Others*	N/A	N/A	-	

				periportal hepatitis and moderate, multifocal lympho-histiocytic vasculitis • Severe diffuse, necrosuppurative bronchopneumonia; moderate, diffuse, fibrinous pleuritic					
CP_28	2014	9 m	Unknown	Moderate respiratory distress	Swab	N/A	+	N/A	-
CP_134	2015	3 m	Male	Moderate respiratory distress	Swab	N/A	+	N/A	-
CP_144	2015	2 y	Female	Severe respiratory distress	Swab	N/A	+	N/A	-
CP_182	2014	3 m	Male	Moderate respiratory distress	Swab	N/A	+	N/A	-
CP_188	2014	2 m	Female	Moderate respiratory distress	Swab	N/A	+	N/A	-
CP_191	2014	6 m	Male	Mild respiratory distress	Swab	N/A	+	N/A	-

Abbreviation: LN, lymph node; m, male; y, year; N/A, no data available; NSA, no sample available; WR, waiting results.

* Other tissues included small intestine, gall bladder, heart and thymus.

3.3 Detection of CanineCV circulating in Thailand by NGS and conventional PCRs

To identify other viral pathogen causing respiratory disease, lung samples of dog no.14P105D and 14P112N were processed for NGS. Over 900,000 trimmed reads were obtained from individual samples; nonetheless, more than 380 and 10 reads were detected from dog no.14P105D and 14P112N, respectively. The highest reads had similarity to canine circovirus, while no other pathogenic viruses were evident. However, the gaps of NGS-derived CanineCV sequences were remained. Those gaps were therefore filled by conventional PCR using primer designed from the NGS output. The detected CanineCV derived from both dogs was confirmed by the PCR and named as CanineCV_14P105D/TH2014 and CanineCV_15P112N/TH2015. Moreover, out of eleventh lung samples derived from 11 respiratory suffering dogs that were subjected for routine necropsy, only dog no.15P061D was positive for CanineCV-specific PCR.

Six respiratory swabs, CP28, CP134, CP144, CP182, CP188 and CP191, out of 20 respiratory swab samples, were positive for CanineCV by using CanineCV-specific PCR. The nearly complete genomes of CanineCV from the swab samples were designated as CanineCV_CP28/TH2014, CanineCV_CP134/TH2015, CanineCV_CP144/TH2015, CanineCV_CP182/TH2014, CanineCV_CP188/TH2014, CanineCV_CP191/TH2014.

3.4 Genome organization, phylogenetic and recombination analyses of CanineCV detecting in Thailand

The coding sequence of all CanineCV Thai strains contained 2 main open reading frames (ORFs), which were coding putative replication associated protein (Rep) and putative capsid protein (Cap). The Rep gene, nt 1- 912, encoded 303 amino acids and the Cap gene, nt 1,116- 1,928, encoded 270 amino acids. Moreover, one additional ORF, ORF3, locating at nt 470-889 in the antisense of ORF1 was also observed (Fig. 21.). Pairwise distances revealed that the CanineCV Thai strains had genetic different ranging from to 10.5 -16.2% compared with the CanineCV UCD3-478 and AZ5586-13 strains. Interestingly, even most of other CanineCV strains detecting in this study were quite similar to each other and related to CanineCV_UCD3-478 strain, the CanineCV CP_191/TH2016 strain was related to JZ98/2014 strain as described in Table 16.

Phylogenetic analysis of CanineCV detecting in Thailand was constructed based on nearly complete sequences, suggesting the most of CanineCV Thai strains were felt in new the clade of CanineCV and closely related to CanineCV UCD3-478 strain (Fig. 22); however, the CanineCV strain CP191/TH2014 was clustered and closed to JZ98/2014 strain. The individual Rep and Cap genes were used to construct the phylogenetic tree, revealing CanineCV strains detecting in Thailand were clustered with CanineCV UCD3-478 for both Cap and Rep; however, the obtained phylogenetic trees revealed discrepancies patterns as shown in Figs. 23-24.

All of CanineCV Thai strains, excepted CanineCV CP191 TH/2014, were identified as recombinant strains by using the RDP4 software. The recombination events in these strains were supported by statistically measuring recombination assays including RDP, GENECONV, BootScan, Maxchi, Chimaera, SiScan, 3Seq and LARD at average *p*-value = 1.42×10^{-4}. Among them, the CanineCV UCD3-478 and JZ98/2014 strains were identified as a major and minor parent, respectively.

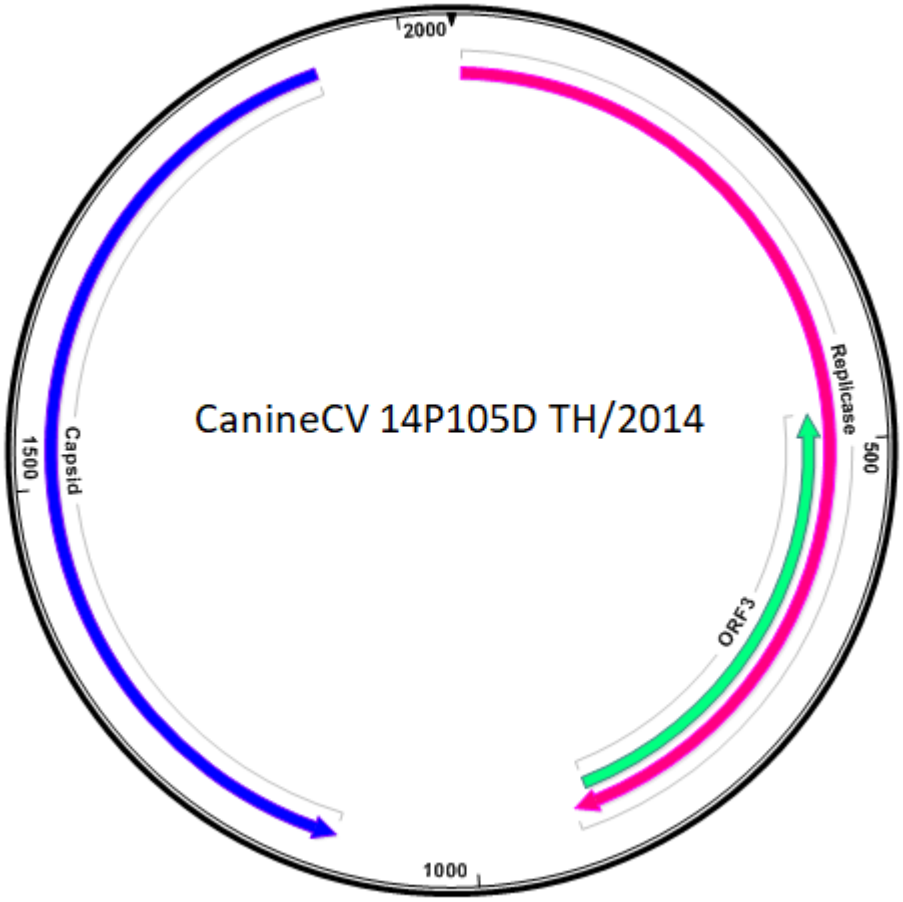
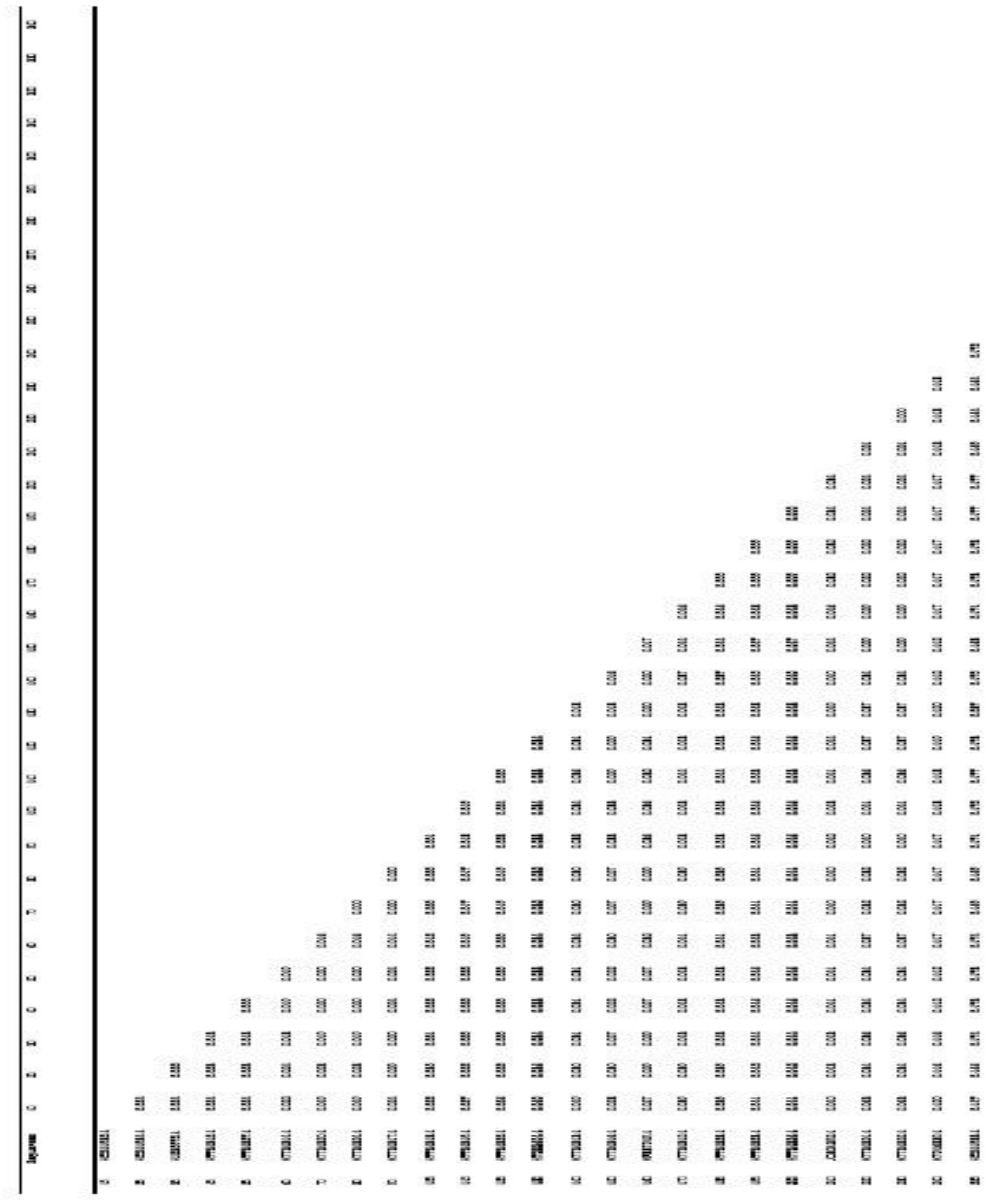


Figure 21. Genome organization of canine circovirus strain 14P105D TH/2014.

Table 16. Pairwise distances of CanineCV Thai genomes compared with other published CanineCV strains.



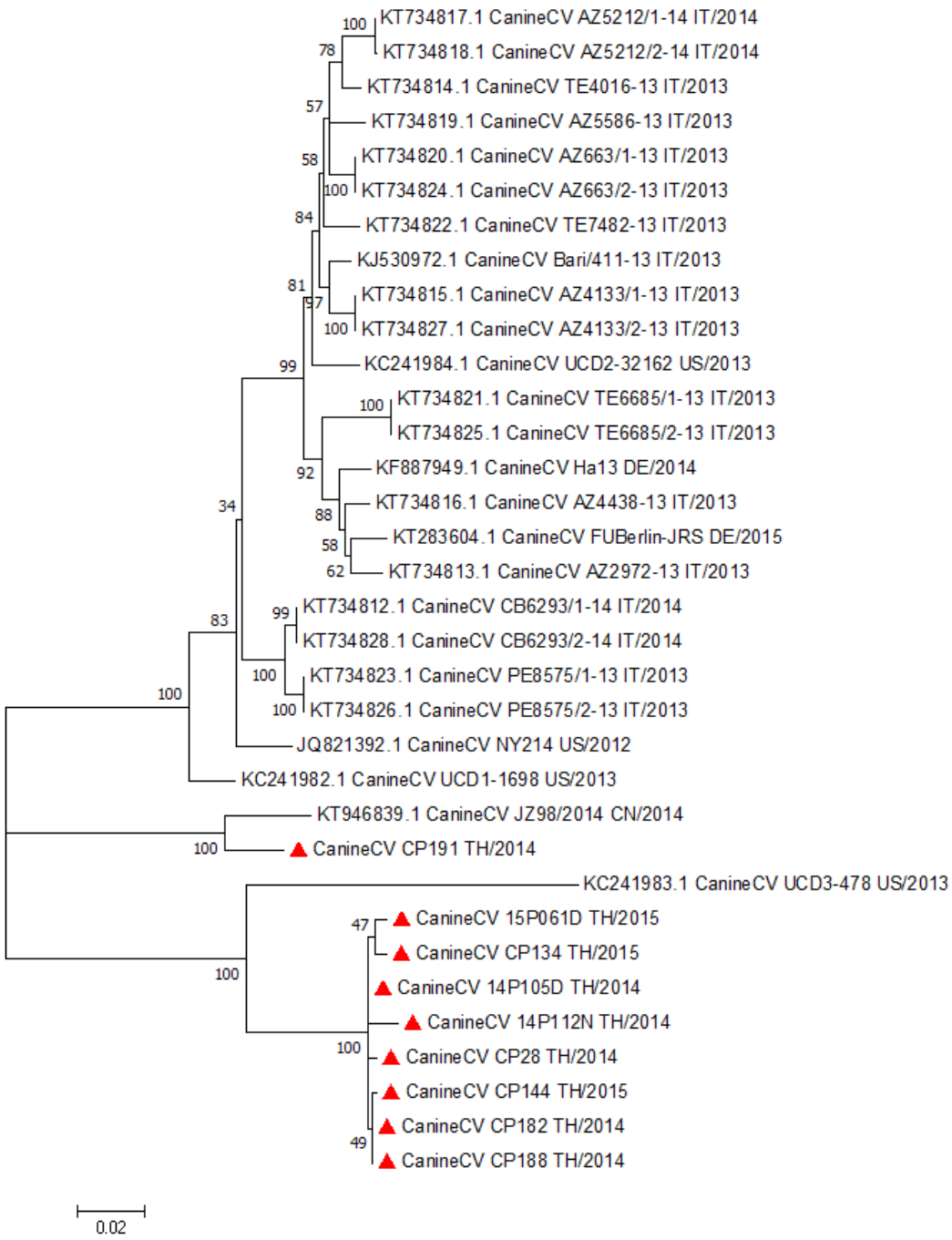


Figure 22. Phylogenetic tree of complete CanineCV genome detecting in Thailand, constructed based on nearly complete sequences of published CanineCVs, bootstrap 1000.

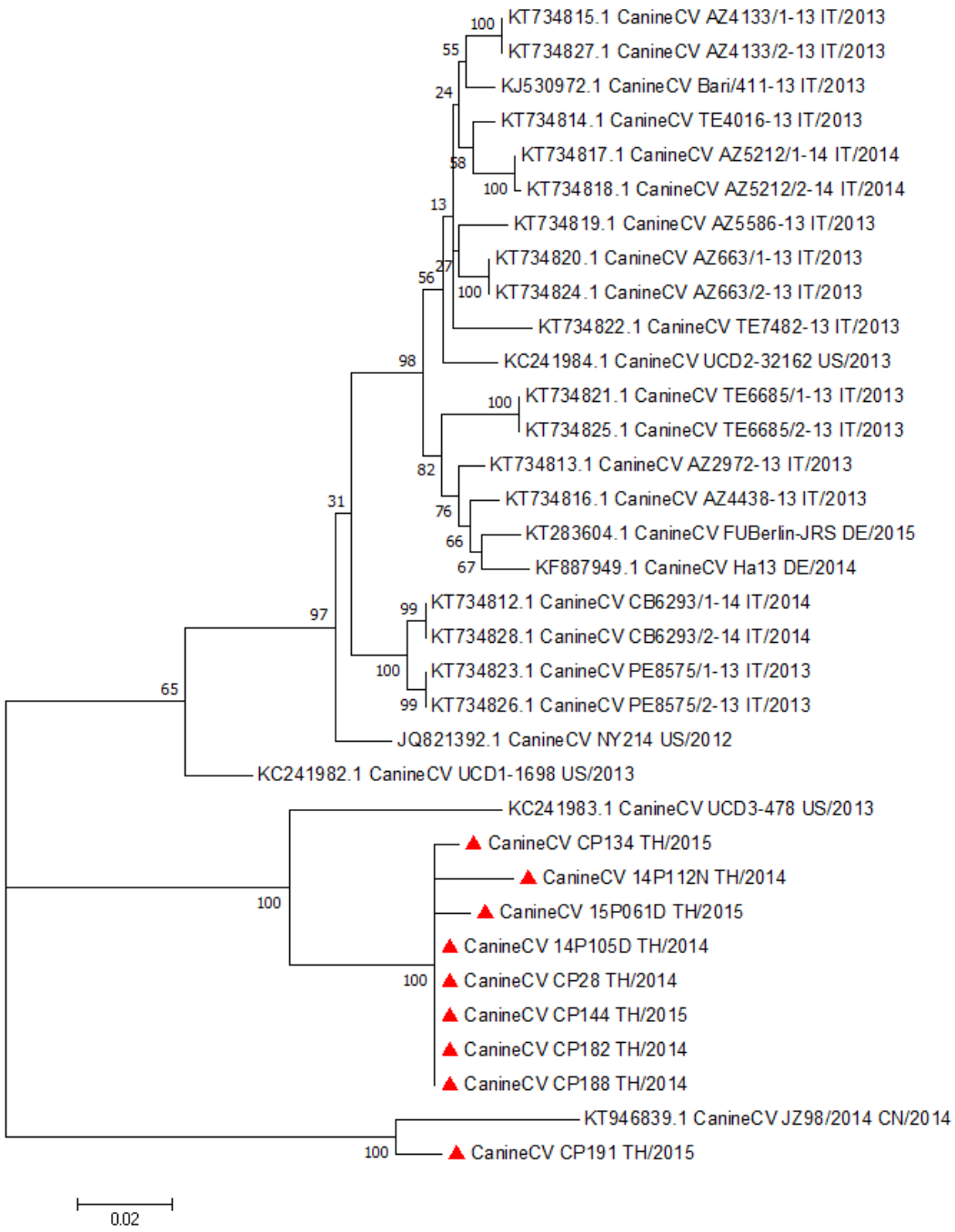


Figure 23. Phylogenetic tree of CanineCV genome detecting in Thailand, constructed based on capsid gene sequences of published CanineCVs, bootstrap 1000.

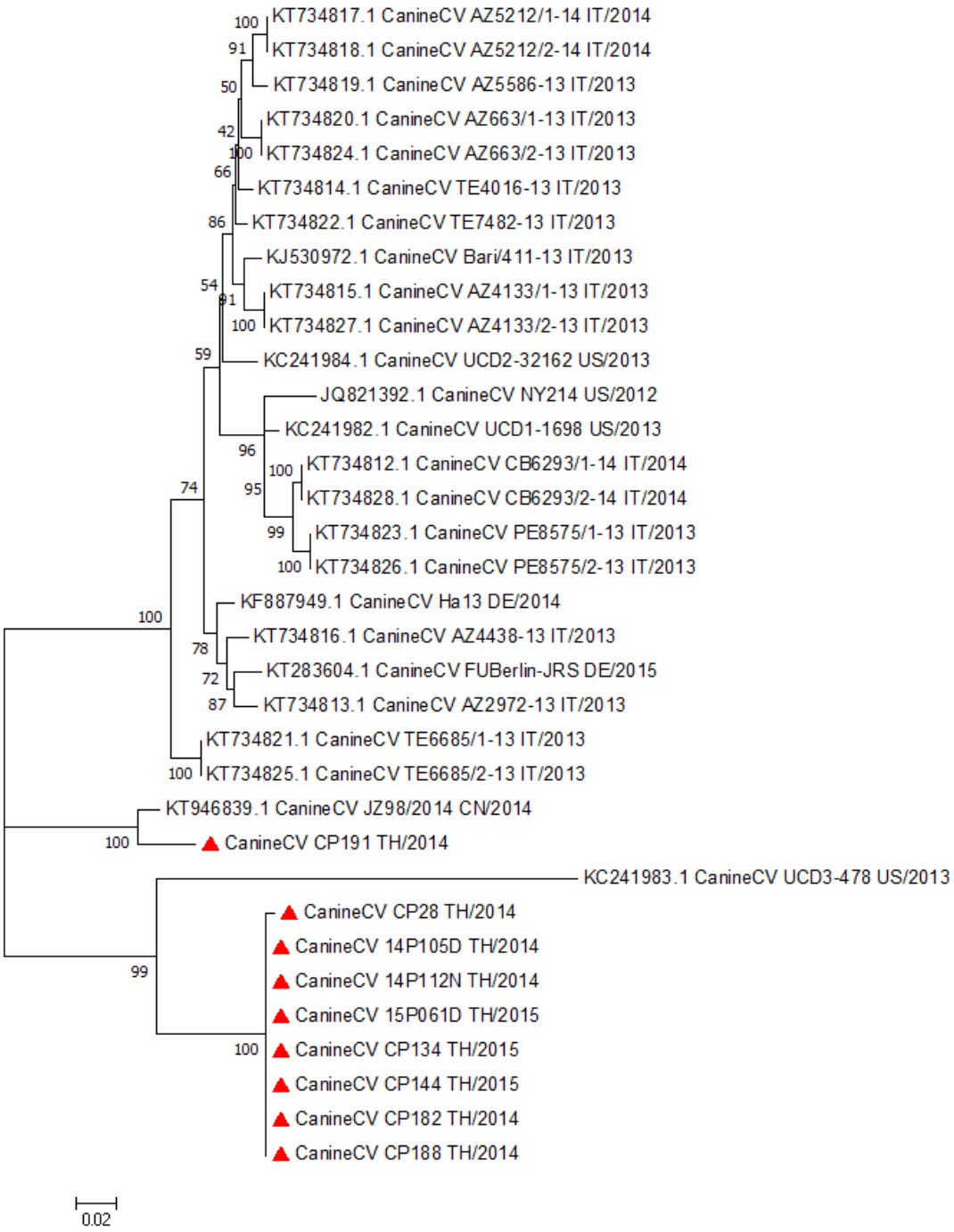


Figure 24. Phylogenetic tree of CanineCV genome detecting in Thailand, constructed based on replicase gene sequences of published CanineCVs, bootstrap 1000.

Moreover, to integrate the results obtained from RDP analysis, potential recombination breakpoint was achieved from SIMPLOT software. The results obtained from this application suggested that the CanineCV Thai strains, excepted CP191 strain, were potential recombinants. The similarity plot generating for CanineCV Thai strains revealed that these strains had high nucleotide identity to a CanineCV UCD3-478 strain (pink line) in the initial Rep region and a whole region of Cap gene; however, the CanineCV Thai genomes showed obviously nucleotide similarity to CanineCV JZ98/2014 strain (red line) at a nearly middle portion of Rep gene (Fig. 25). Moreover, the bootscan analysis also confirmed the result obtained from disparities in phylogenetic origin of CanineCV Thai strains. Potential recombination breakpoint of the CanineCV Thai strains was derived from CanineCV JZ98/2014 that incorporated within nt. 380-820, locating in Rep gene, despite the CanineCV UCD3-478 served as a parental template for Cap gene (Fig. 26). Data achieved from RDP and SIMPLOT was similar.

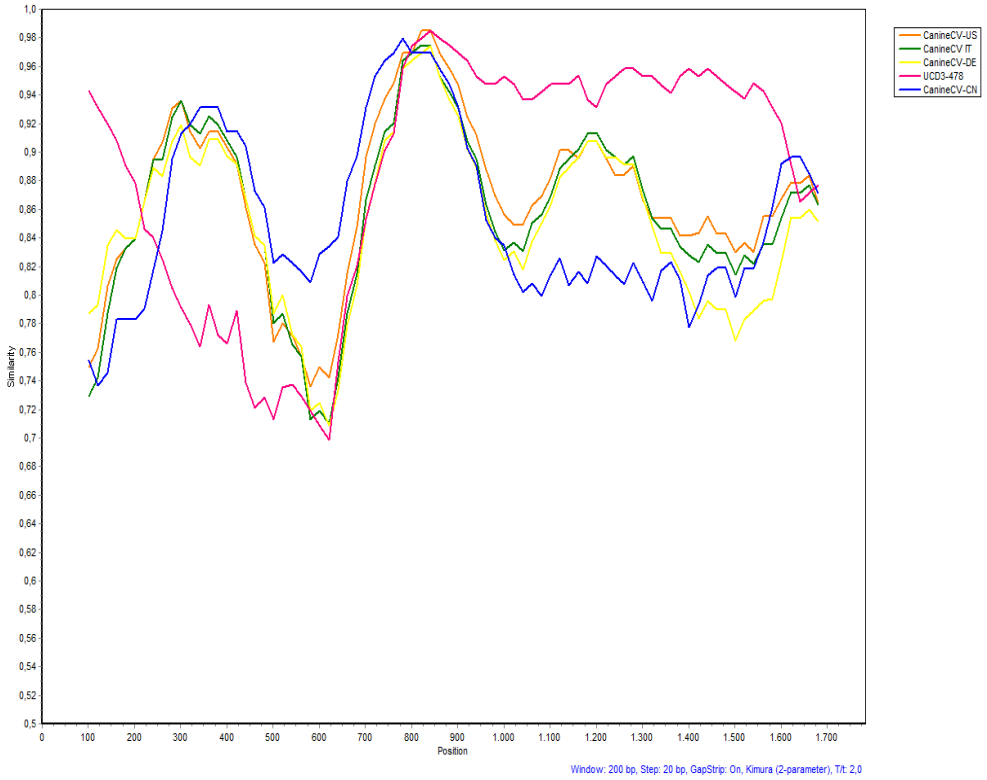


Figure 25. Similarity plot of CanineCV Thai strain (query), compared with published CanineCV in various strains, showing potential recombination events. SimPlot.

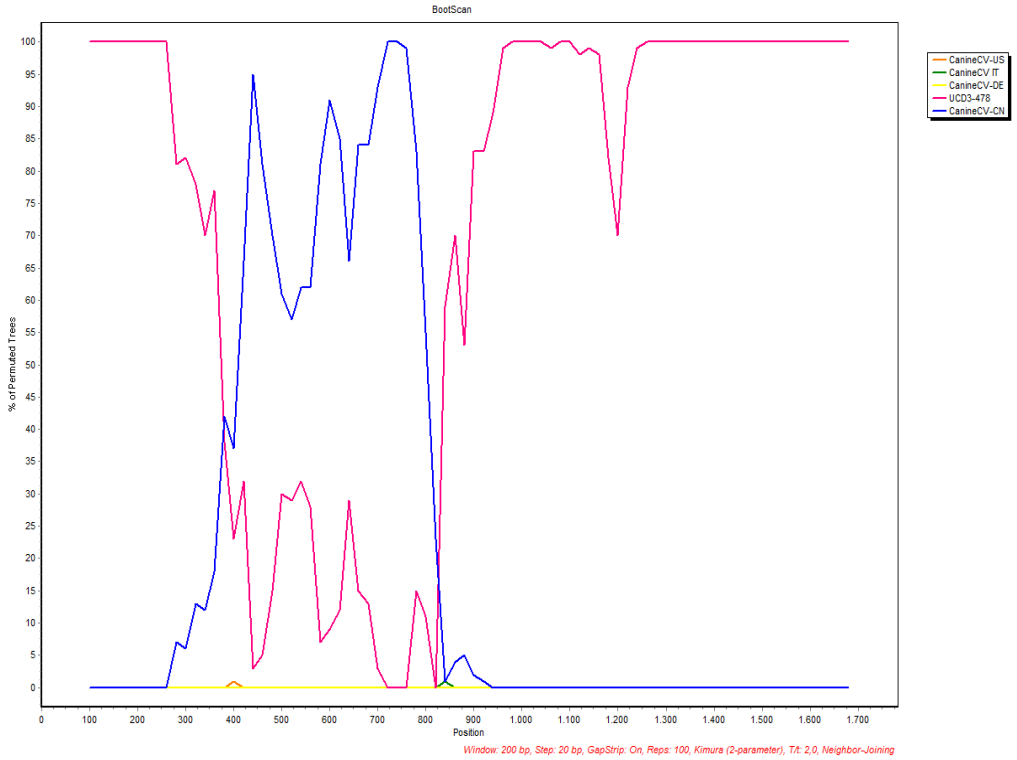


Figure 26. Boot scan analysis of CanineCV Thai strain (query), compared with published CanineCV various strains, suggesting potential recombination in Rep gene. SimPlot.

Sequence comparison of potential recombinants, CanineCV Thai strains with their parents

Additional information of genetic recombination breakpoints was achieved by differentiation of genetic marker positions between recombinants and their parenteral lineages. Because of genetic similarity among CanineCV Thai strains, 14P105D TH/2014 strain was used as a represented sequence for analysis (Table 17.). The genetic markers were identified in the CanineCV 15P061D TH/2015 by nucleotide marker position starting from nt383 through nt807, which was incorporated by nucleotide derived from CanineCV JZ98/2014 isolate. This analysis also confirmed the predicted nucleotide recombination break points as shown as in RDP and SIMPLOT analyses. Moreover, the CanineCV CP191 TH/2014 strain was also analyzed and shown that it was most

exclusively evidenced by the JZ98/2014 isolate; however, mutant nucleotides were detected in nt 333, which only observed in two Italian CanineCV strains (AZ4133 1/13).

Table 17. Comparison of nucleotide marker positions between recombinant CanineCV 14P105D TH/2014 and its parenteral CanineCV strains.

Genome Position	UCD3-478	JZ98/2014	14P105D TH/2014	CP191TH/2014
Rep gene				
327	A	G	A	G
333	C	G	C	T*
339	T	A	T	A
345	A	G	A	G
383	C	A	A	A
384	T	A	A	A
390	G	A	A	A
408	T	C	C	C
414	G	C	C	C
426	C	T	T	T
456	A	C	C	C
525	C	G	G	G
540	G	C	C	C
543	C	T	T	T
692	G	T	T	T
762	A	C	C	T
805	A	G	G	G
807	T	G	G	G
825	T	C	T	T
837	T	C	T	C
1095	G	T	G	T
1096	T	G	T	G
		Cap gene		
1125	T	G	T	T
1182	G	C	G	C
1305	T	C	T	C
1329	A	T	A	T

* Specific nucleotide mutation detected in CanineCV CP191 TH/2014 strain.

3.5 CanineCV genome distribution and in situ hybridization

CanineCV-specific PCR was successful to detect the virus genome in lung, liver and tracheobronchial lymph node in all dogs. CanineCV nucleic acid was also detected in tonsil of dog no. 14P015D and 14P112N, whereas it was detected in kidneys of dog no.15P061D. However, the brain tissue of all dogs was negative (Table 15.). *In situ* hybridization revealed single weakly positive cell in a lymph node and numerous positive macrophages within the follicle center of the tonsils from dog no. 14P105D (Figs. 27-29). Other tissues including lung, liver, spleen, intestine and kidney from all three animals were negative.

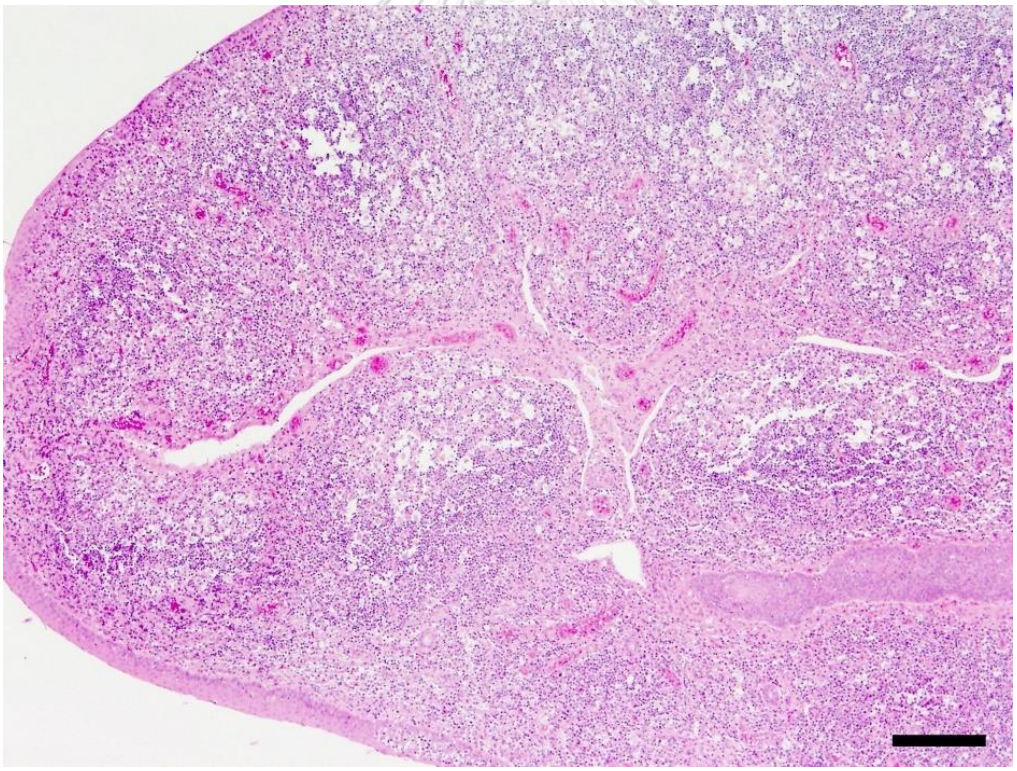


Figure 27. Canine Circovirus infection, dog, tonsil. Mild lymphoid hyperplasia was noted in dog no. 14P105D, 40X, H&E.

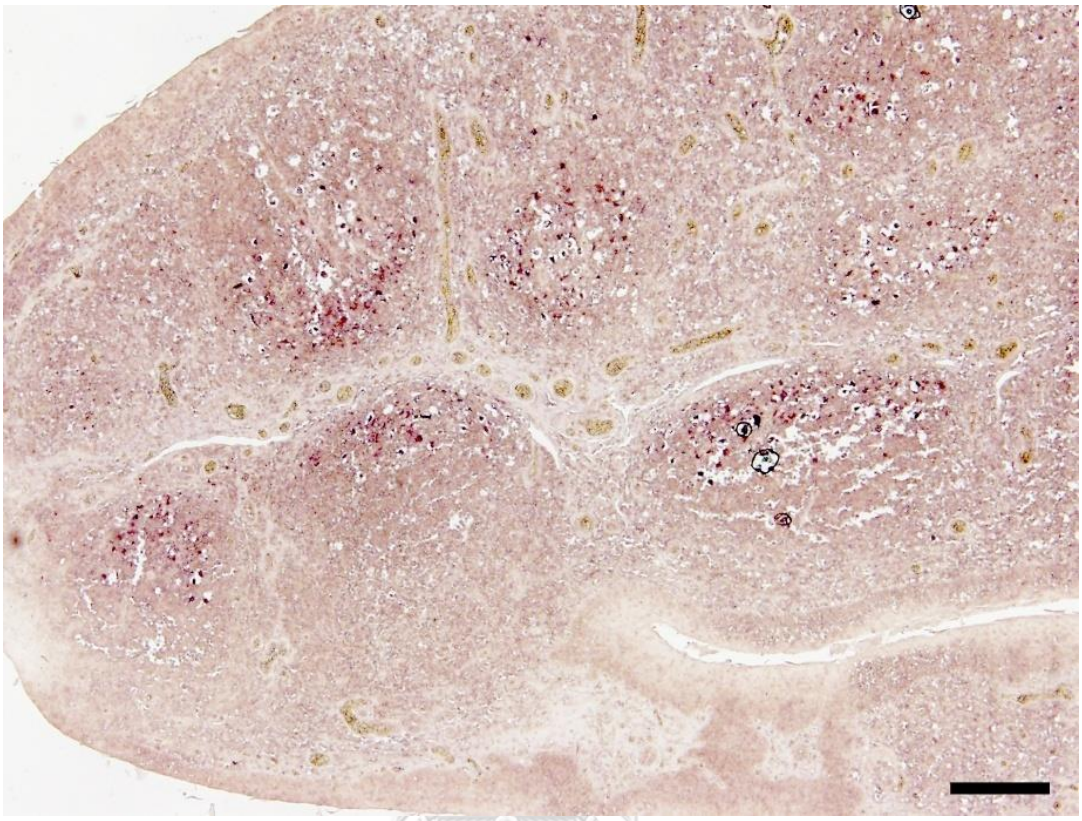


Figure 28. Canine Circovirus infection, dog, tonsil. Strong positive signals of in situ hybridization were detected in hyperplasia tonsil of dog no. 14P105D, 40X, In situ hybridization (ISH).

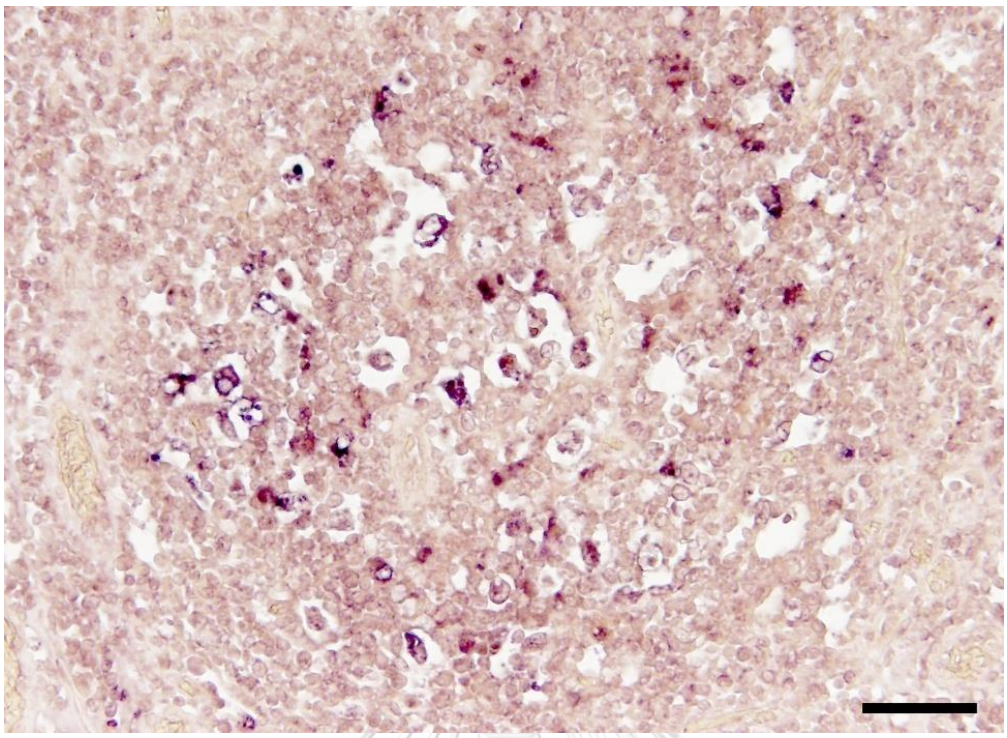


Figure 29. Strong positive signals of in situ hybridization were detected in cytoplasm of macrophages located in tonsil of dog no. 14P105D, 100X, ISH.

DISCUSSION

Recently, novel canine viruses have been investigated and hypothesized as a possible cause for many diseases and syndromes. The CanineCV, a novel non-porcine circovirus, was firstly established and associated with vasculitis and hemorrhagic syndrome (Li et al., 2013a). Several studies also detected the CanineCV genome from intestinal samples and attempted to search an association of the CanineCV with intestinal disease (Anderson et al., 2017; Decaro et al., 2014; Hsu et al., 2016; Li et al., 2013a; Thaiwong et al., 2016). However, clinical association and pathogenic role of CanineCV infection are still undetermined. In this study, we aimed to discover an associated pathogen(s) from dogs suffering from acute respiratory disease and gained negative PCR result from routine respiratory virus detections. Interestingly, the CanineCV genome was discovered from dogs' lungs with respiratory lesions by using

NGS. This finding prompted us to investigate further the distribution of CanineCV in other organs, as well as, in other respiratory samples from illed dogs by using specific PCR and *in situ* hybridization. In this present study, the CanineCV genome could be detected in lung, liver, tonsil, tracheobronchial lymph node and kidneys that were consistent with previous findings (Li et al., 2013a; Zaccaria et al., 2016). However, several remaining tissues of CanineCV-infected dogs were limited such as intestinal tract and resulted in the fragmentary of viral distribution data. Nonetheless, tissue localizations of CanineCV confirming by ISH showed only in cytoplasm of macrophages locating in tracheobronchial lymph nodes and tonsil. No CanineCV-ISH signal was detected in other tissues, regardless of the evidence of tissue inflammation by histopathology. Consistent with our findings, the CanineCV has been detected in macrophages of lymphoid follicles locating in intestine, spleen and liver even though the virus genome could be detected in other organs by using PCR (Li et al., 2013a; Thaiwong et al., 2016; Zaccaria et al., 2016). These results might be associated with the hematogenous spread of CanineCV, and then the virus was detected in several organs. The hypothesis of viral hematogenous spread was supported by the evidence of CanineCV detection in dog sera (Kapoor et al., 2012a; Li et al., 2013a). Based on our knowledge, clinical presentations and pathological features were varied among CanineCV-positive dogs. In particular, CanineCV-infected dogs as well as PCV-2 infected pigs exhibited resembling histological features such as granulomatous or necrotizing lymphadenitis (Allander et al., 2001; Chae, 2005; Li et al., 2013a). Moreover, the co-infection with other pathogens were frequently presented with natural CanineCV or PCV-2 infection in their respective hosts (Anderson et al., 2017; Hsu et al., 2016; Li et al., 2013a; Opriessnig and Halbur, 2012; Thaiwong et al., 2016; Zaccaria et al., 2016). Our study showed that CanineCV-infected dogs were co-infected with other pathogens, supporting the assumption that the *Circoviridae* virus could alter immune responses and lead to immunosuppresion (Li et al., 2013a; Opriessnig and Halbur, 2012). However, the role of co-infected pathogens in these infected dogs needed to be further clarified.

Analysis of nearly complete genome of CanineCV detecting in Thailand was conducted and showed that they were clustered into two clades. Most of CanineCV

Thai strains were grouped into clade that closely related to CanineCV-UCD3-478 strain, isolated from serum of an American dog; however, CanineCV strain CP-191/TH2016 was closely related with CanineCV JZ98/2014 strain, isolated from a dog in China. These findings suggested that CanineCV strains circulating in Thailand have genomic variations. Surprisingly, pairwise distance analysis of CanineCV Thai strains illustrated at least 10.5% genetic difference from closed-related strain even though they were grouped in the same clade; this result suggested our CanineCV are the novel strain. Preliminary analysis of CanineCV Thai strains revealed discordant phylogenetic relationship with other CanineCV strains. For example, the CanineCV Thai strains were clustered together with CanineCV UCD3-478 when compared by complete genome and Cap gene; however, they were differently grouped as a new clade with CanineCV UCD3-478 strain when constructed the phylogenetic tree of Rep gene. Paradoxical results of those phylogenetic trees were implied to possible potential recombination of CanineCV Thai strains. According to viruses in family *Circoviridae*, potential naturally genetic recombination events were reported in beak and feather virus (Heath et al., 2004), Torque teno virus (Manni et al., 2002), and PCV, mammalian circoviruses, which presented the genetic recombination among PCV2a and –b, as well as in many other strains of PCV2 (Hesse et al., 2008; Ma et al., 2007). These findings suggested the possibility of natural recombination events occurring in other circoviruses. In this study, by integrated several platforms for recombination detection i.e. boot scanning, phylogenetic incongruity, genetic marker identifications and many sequence analyses, we were able to firstly identify the recombination breakpoint in novel CanineCV Thai strains at base position 380-820, locating in Rep gene and overlapping the ORF3 region. Due to the lack of information regarding genomic function of CanineCV, we supposed that genomic function of the PCV might be useful for further discussion in this study. The Rep gene, encoded by ORF1 and commonly found in many circoviruses, plays a crucial role for viral replications (Cheung, 2006; Finsterbusch et al., 2005; Mankertz and Hillenbrand, 2001), while the ORF2 encoded the immunogenic icosahedral capsid protein (Lekcharoensuk et al., 2004; Nawagitgul et al., 2000). Studies of PCV2 genomes revealed that the viruses could undergo recombination contributing for genetic

diversity of PCV2 strains and supported that the Rep gene was the best region to observe the recombination events (Olvera et al., 2007; Ramos et al., 2013), which found in many researches (Hesse et al., 2008; Kim et al., 2009; Ma et al., 2007). However, various reports also demonstrated the intra- and inter-genotypic recombination events in the Cap gene (Cadar et al., 2012; Cai et al., 2012). These studies might support our focuses that relied on the recombination event occurred in the Rep gene of CanineCV. The ORF3, an overlapping anti-directional region of ORF1 gene, was firstly recognized in this novel CanineCV Thai strains. Normally, ORF1 gene is responsible for virus-induced apoptosis as a report in PCV2 (Liu et al., 2005) and also serves as a dispensable region for virus replication (Juhan et al., 2010b). Recent studies showed that ORF3-deficient PCV2-infected mice showed less severity than mice infected with wild type strains (Liu et al., 2007) but the evidence of pathogenicity of ORF-3-deficit PCV2 infected pigs was limited (Juhan et al., 2010b). Therefore, function of the ORF-3 region as well as an altering mutation in this region of any *Circoviruses* should be comprehensively determined. Even though, previous studies of CanineCV genome has already been described the ORF3, but it did not take into account (Li et al., 2013a). Interestingly, our study showed that the recombination breakpoint occurred in Rep gene of CanineCV Thai strains, which was intersected with the ORF3 region. This finding might shed a light on the role of recombination event that probably alter the pathogenicity and virulence of CanineCV. Further investigation of the ORF3 function is definitely indeed for elucidation the ability of CanineCV infectivity and replication.

In conclusion, data gained in this study contributes to a novel insight and better knowledge of the emergent CanineCV in a regional context showing the existence of novel recombinant CanineCV strains reported in Thailand. The genetic study and phylogenic analysis based on only single genome region might be misinterpreted because of genetic recombination of the CanineCV. Continuous investigations of the virus evolution resulted from either mutation or recombination or both are essential for the virus evolutional knowhow. Study regarding the function of each region of CanineCV genome might contribute the better understanding of CanineCV pathogenesis in the future.

CHAPTER 3

3.1 GENERAL DISCUSSION AND CONCLUSION

Canine infectious respiratory disease complex (CIRDC) is an important disease that impacts on dogs, especially puppies or immunosuppressed dogs, and is frequently associated with viral infections. It has gained attention recently, because many viruses have been discovered and co-infections with multiple pathogens are often fatal. Thus, the development of diagnostic tools for CIRDC associated virus detection is necessary to enhance the diagnosis coverage. In this study, multiplex RT-PCR and multiplex PCR assays for the detection of CIRDC-associated RNA and DNA viruses, respectively, were developed and compared with conventional methods. Both developed multiplex PCRs could detect several CIRDC viruses efficiently. The two multiplex PCRs gave similar results equivalent to that obtained from the conventional simplex PCRs which required six separate reactions per sample. Although multiplex PCR has been developed previously to detect several pathogens of CIRDC, such as CIV, CDV and CRCoV (Jeoung et al., 2013), its application remained limited because of the narrow range of viruses covered, with other CIRDC-associated viruses being neither detected nor ruled out. Thus, our study provides a broader range of CIRDC virus detections including CIV, CDV, CRCoV, CPIV, CaHV-1, and CAdV-2. (CAdV-2). The overall sensitivity of the multiplex RT-PCR and multiplex PCR was more than 90% and 87%, respectively, compared to their simplex counterparts. However, the detection of CRCoV was modified as a hemi-nested RT-PCR to increase its sensitivity (Poovorawan, personal communication). The false negative reactions when performing multiplex PCRs in this study might be resulted from the selection of the single optimized annealing temperature (Ta) for several primer pairs and the low amount of particular target genes (Bellau-Pujol et al., 2005). These might be resulted in the decreased sensitivity of the developed multiplex PCRs. Moreover, there was 100% specificity in both modalities for clinical sample detection.

When we evaluated the commercially available three-antigen rapid test kit (CAdV-2, CIV and CDV) compared with PCR assays, we found only CIV detection showed

an unexpected sensitivity and specificity. A previous study reported that the developed multiplex RT-PCR for H3N2 CIV, CDV and CRCoV detection had an almost 100% sensitivity and specificity compared with the conventional RT-PCR and rapid antigen test kit (Jeoung et al., 2013). In contrast, our study showed that the CIV-positive samples by multiplex RT-PCR were negative when tested with the rapid antigen test kit. Our findings were consistent with previous investigations reported that many rapid test kits might have a low sensitivity to detect the influenza virus, but could still be suitable for rapid in-house clinical applications (Liao et al., 2011; Pecoraro et al., 2013). The controversial results may resulted from the different test kit, viral copy number, duration of sample storage, route of sample collection, and various virus strains; all factors can influence the test results (Banoo et al., 2010).

Interestingly, in this study revealed about 70% (71/102) of samples from the clinical respiratory illness dogs showed multiple CIRDC virus infections. This finding supports the fact that CIRDC is a complex disease and usually co-infects with more than one pathogen. Recently, Jeoung et al. (2013) applied both nasal swab and whole blood samples for CIRDC virus detection; they found that only CDV, but not CIV and CRCoV, could be detected from the whole blood sample (Jeoung et al., 2013). Correspondingly, respiratory swab has been considered to be the appropriate sample for the detection of respiratory pathogens (Gritzfeld et al., 2011; Posuwan et al., 2010). Therefore, in our study, swabs from nasal passage and oropharyngeal area served as the suitable samples, and because of their ease and noninvasive technique, and swabs directly applied on the viral shedding routes. This study also suggested that the sampling sites are important depending on the type of virus. The CAdV-2 and CaHV-1 mostly replicate in the lower respiratory tracts and shed via respiratory discharge, consisting with our findings that they were mostly detected in the oropharyngeal swabs, even though the nasal swabbed samples could often detect these viruses as well. However, the CAdV-2 primer pair used in this study was able to amplify CAdV-1 DNA virus which also shows airborne transmission and replicates in tonsil (Buonavoglia and Martella, 2007). Therefore, the positive PCR reaction for canine adenovirus could not discriminate between CAdV-1 and CAdV-2 in this study. Additionally, CaHV-1 can

be latent in various nerve ganglions, resulting in negative results from nucleic acid-based CaHV-1 detection in respiratory discharges in non-symptomatic dogs (Miyoshi et al., 1999).

In this study, 3 out of 15 vaccinated dogs receiving, at least once, combined vaccine against CPIV, CDV and CAdV-2 showed PCR positive results for CIRDC virus detection (2 CDV positive dogs and 1 CPIV positive dog). Even though live attenuated vaccines can give false positive results with molecular testing, it is essential to discriminate between wild-type infection and recent vaccination for the prevention of false positivity in the future. This study documented CaHV-1 and CRCoV circulation in Thailand for the first time. In 2012, CIV H3N2 was discovered in Thailand from dogs with flu-like symptoms (Bunpapong et al., 2014). Here, CIV and CRCoV were the most frequently detected viruses in CIRDC-infected dogs, suggesting that the viruses might spread rapidly. These viruses were not only found in single infections, but they were also found as co-infections together or with other viruses. This study also exhibited a higher level of infections compared with a previous report (Pecoraro et al., 2013), although this might be caused by the different timing of sample collection, population size and locations. However, it has previously been reported that infection with CRCoV and CPIV might facilitate or initiate the disease and, subsequently, enhance the entry of other pathogens (Erles et al., 2004), so the prevalence of infected dogs is then increased. Moreover, we found that the dogs that were infected with CIV, CPIV, CDV and CRCoV showed a greater severity of clinical symptoms, such as marked bronchopneumonia and sudden death (data not shown). This finding is consistent with other investigations suggesting that co infections might augment the severity of clinical symptoms (Erles et al., 2004; Posuwan et al., 2010). Thus, advanced genetic-based detection methods, such as multiplex PCR assays, are considered as an alternative diagnostic platform for a panel of suspected CIRDC causing viruses with a high sensitivity and specificity. Because of the cost benefit and practical usage, the developed multiplex PCR assays are suitable for a screening test for disease diagnosis, quarantine and prevention measures, especially in developing countries.

Commonly, The CIRDC is associated with environmental factors, individual host susceptibility and infectious pathogens, which they are primarily viruses (Buonavoglia and Martella, 2007; Priestnall et al., 2014). The CIRDC viruses are commonly detected in dogs with respiratory problems and are endemic in poor conditioned dogs, overcrowded shelters, hospitals and pet grooming centers (Erles et al., 2004; Kawakami et al., 2010; Monteiro et al., 2016; Schulz et al., 2014; Weese and Stull, 2013). A hospital associated infection compared with community infection is not well established. The epidemiology of CIRD infected dogs has been reporting in many years, but represented contrasting results (Erles et al., 2004; Schulz et al., 2014; Viitanen et al., 2015). In this study, we focused on the prevalence of CIRDC virus detections in terms of community-acquired infection (CAI) and hospital-associated infection (HAI) that are supposed to be the factor of CIRDC infections. The HAI-diseases, especially respiratory tract infections, have been considered as a risk for nosocomial transmission, but they have not been investigated in clinically infected dogs (Kawakami et al., 2010; Weese and Stull, 2013). There has been increasing concern about infections due to emergence of multidrug resistant pathogens as well as novel pathogens that are transmitted from other hosts (Weese and Stull, 2013).

In this study, all six viruses were detected in both sample groups of CAI and HAI, suggesting that these viruses might either be presented or disseminated in both environments. Moreover, we found that CRCoV and CIV were highest prevalence in the CAI and HAI dogs, respectively. This finding was in contrast to previous observations found that CPIV was the most detected virus in CIRDC dogs(Erles et al., 2004; Schulz et al., 2014; Viitanen et al., 2015), although this might be influenced by various factors, including the different geography, vaccination strategies and hospital hygienic management. This study showed that CDV was significantly detected in CAI dogs than that in HAI counterpart. In Thailand, there are many unvaccinated free-roaming dogs that could serve as a reservoir for CDV dissemination in CAI dogs (Posuwan et al., 2010; Radtanakatikanon et al., 2013).Meanwhile, other CIRDC viruses were detected in both the CAI and HAI dogs without any statistically significant difference in the frequency of occurrence between them. This is in accord with a recent study reported that there

was no statistically significant difference in CPIV, CAdV-2 and CRCoV infections between dogs from private households and dogs from shelters or kennels (Schulz et al., 2014). Similar to other investigations focusing on HAI-derived CIRDC endemics, we found all six CIRDC viruses were detected in the HAI dogs (Kawakami et al., 2010; Weese and Stull, 2013). This might imply that these viruses already are endemic or spread in animal health care centers, such as hospitals or pet grooming centers.

The prevalence of CDV, CPIV, CAdV-2, CaHV-1 and CIV circulation in Thailand has been documented (Bunpapong et al., 2014; Piewbang et al., 2016, 2017; Posuwan et al., 2010), but not for that of CRCoV. This study, therefore, is the first report of CRCoV infected dogs in Thailand. The most commonly detected viruses were CIV and CRCoV (> 50%) in both CAI and HAI groups that is in contrast to previous reports that CPIV is the major CIRDC virus found in dogs with respiratory disease in Asia (Mochizuki et al., 2008; Posuwan et al., 2010) and Europe (Decaro et al., 2016; Erles et al., 2004; Schulz et al., 2014; Viitanen et al., 2015).

In the present study, 81.2% (108/133) of CAI and 78.9% (60/76) of HAI dogs were multiple virus detections, supporting the complex condition of this respiratory disease, which is often a co-infection rather than a single pathogen. We also observed that the majority of multiple CIRDC viruses were co-detected with either CIV, CRCoV, or both; however, this observation does not allow interpretation as to whether they are primary and/or secondary pathogens. The CRCoV and CIV usually induced mild clinical symptoms by interfering with the respiratory defense mechanism and so leading to super-infections with other pathogens. Moreover, co-infection of CIV and CRCoV may be synergistic and lead to severe tracheobronchitis.

There were no significant differences in the sex, age and vaccination status of dogs between single and multiple virus detections. This finding need to be awareness for interpretation because of no significant difference between vaccinated- and unvaccinated dogs. The commercial vaccine used in Thailand against only CPIV, CDV and CAdV-2 but not for others. Thus, the multiple infections may be caused by the other viruses, which have no commercial vaccine available, and may affect the study results by allowing infection with other viruses in the vaccinated dogs. However, there

were different levels of single and multiple virus detections between vaccinated and unvaccinated dogs. The vaccinated dogs had a lower proportion of both single and multiple detections compared with the unvaccinated dogs. Interestingly, there was significant difference in CIRD-affected age groups when compared with clinical severity level. CIRD-affected puppies had more severe clinical level compared with other age groups. Because host susceptibility such as premature immune response and unvaccinated dogs might be explained in this finding. However, there was no significant difference of clinical severity level between CAI and HAI dogs which is inconsistent with our hypothesis.

Significant association between CIRD agents and clinical respiratory scores was observed in double detection of CIV and CRCoV. This co-detection represented the highest proportion and most often found with other CIRD viruses. These observations showed that CIV and/or CRCoV can be either primary or secondary agents that commonly found co-infected with others; however, bacterial infection was not ruled out in this study. Many investigations have revealed that either viral or bacterial co-infection leads to an increased severity, but some studies have revealed no significant differences in the clinical severity between single and multiple infections (Cilloniz et al., 2012; Decaro et al., 2016; Huijskens et al., 2014; Viitanen et al., 2015).

Because CIRDC is caused by multi-factorial etiologies, changes in the environment, such as climate temperature and amount of rainfall, may affect the host susceptibility to the pathogens. The prevalence of CIRDC infections was seen to increase as the amount of rainfall increased and after the average temperature declined (July–October 2014). Nevertheless, even when the rainfall amount and average temperature increased during January–April 2015 the prevalence of CIRDC infections still slightly increased. However, this finding lack of power to explain the relation of CIRDC detected dogs and any climate changes. Previous studies regarding respiratory viruses in climate zones and tropical countries have documented that infectious respiratory viruses, especially influenza, are usually observed during or immediately after the rainy season. However, the CIRDC infection patterns were not

represented during March–December 2013 because of the limited number of samples in this study.

Since healthy or asymptomatic dogs were not included in this study then the true prevalence of the CIRDC virus infections could not be assessed. Moreover, some of CIRDC virus such as CPIV, CAdV-2 and CRCoV could be detected from healthy dogs, suggesting that some of CIRDC virus may not exhibit respiratory problem; however, individual host susceptibility should be concerned. It would be interesting to study healthy dogs, which might serve as an asymptomatic reservoir for CIRDC infections in the CAI group. Furthermore, the result of CIRDC virus detection by using PCRs form the respiratory swabs could not be totally implied that those dogs were “infected” but only a detection of represent CIRDC pathogens that might associate with respiratory problem. Additionally, samples from the North and North Eastern regions of Thailand were not collected for practical reasons and so the epidemiology could not be achieved in this study. Moreover, since we only focused on six viral pathogens associated with CIRDC, then the role of other pathogens, including known bacterial pathogens, such as *Bordetellabronchoseptica* and *Mycoplasma* sp., and novel viruses, such as CBoV and CnPnV, that might be implicated remains unknown.

Because of novel emerging viruses, recent literatures were conducted by metagenomic analysis to explore the possible pathogens especially virus(es) causing the disease. This prompts us to investigate the viral pathogens causing respiratory problems. In this study, we detected the 2 possible pathogens by next-generation sequencing (NGS) that might be associated with respiratory problem; they were isolated from five ill dogs showing negative results of the developed multiplex PCRs for CIRDC detection.

In the present study, we have investigated the etiology of the deaths of a litter of pups (16P177W-16P179W) that had succumbed to an unknown disease, which was initially suspected to be associated with respiratory failure. Gross- and histopathology did not indicate any pathology that may have led to respiratory failure. Routine diagnostic post-mortem investigations aiming at identifying pathogens known to be associated with canine respiratory disease were also negative. Consequently, the cause

of death could not be determined. This challenged us to look for other viral pathogens by NGS, which resulted in the identification of CBoV-2 in three pup carcasses. Recently discovered viruses of the genus *Bocaparvovirus* have been associated with gastrointestinal and respiratory disease in several mammalian species, including humans and dogs (Bodewes et al., 2014; Choi et al., 2015a; Guido et al., 2016; Schildgen et al., 2008). Based on comparative phylogenetic analyses of the nucleotide sequences obtained, the CBoV TH-2016, our isolate from this study, proved to be a novel strain of CBoV-2, most closely related to previously identified CBoV-2 strains from South Korea and Hong Kong. A unique deletion of 18 nucleotides, located in the VP2 gene of CBoV-2, has been associated with respiratory disease in pups (Kapoor et al., 2012b), while CBoV-2 strain F13000791S, associated with severe enteritis only, did not show this deletion (Bodewes et al., 2014). Concomitantly, this unique 18-nt deletion was evident in the newly identified CBoV TH-2016. However, the significance of this deletion remains to be determined, as no amino acid deletions in the VP2 gene were detected in another recent case of a CBoV-2 infected dog with severe respiratory disease (Choi et al., 2015b). In addition, presence of ORF4 was shown in CBoV TH-2016. Absence of ORF4 downstream ORF1 has been previously associated with respiratory problems (Choi et al., 2015b). In addition, the CBoV TH-2016 strain had a relatively long NS1 region, encoding 793 amino acids. This finding is consistent with a previous study suggesting that the CBoV genome possesses a putative second exon encoding the C-terminal region and conserved RNA-splicing signals closed to the NS1, that may generate a longer NS1 (Lau et al., 2012). Recent reports documented that the longer NS1 region was observed in dogs showing respiratory (Choi et al., 2015b; Kapoor et al., 2012c).

Although bocaviruses are DNA viruses, most of which show limited evolutionary kinetics, many studies have suggested that parvoviruses are capable of rapid evolution resulting in novel genotypes and species, similar to that observed evolutionary dynamics in RNA viruses (Duffy et al., 2008; Nguyen et al., 2002; Servant et al., 2002). Similarly, recent studies have indicated that human and animal bocaviruses, such as human bocavirus (HBoV), porcine bocavirus (PBoV) and feline bocavirus (FBoV), readily

undergo genetic rearrangement and recombination (Hoelzer et al., 2008; Hogan and Faust, 1986; Kapoor et al., 2010; Kapoor et al., 2009; Lau et al., 2012; Lau et al., 2011). Based on phylogenetic analysis of whole genome and VP1/2 sequences, CBoV TH-2016 was shown to share the closest evolutionary relationship with CBoV-2 strains detected in South Korea. However, analysis of the NS1 region alone indicated that this strain was most closely related to those described in Hong Kong. The VP1/2 and NS1 genes of CBoV-2 are highly variable regions that have been associated with viral virulence. Several researches in HBoV also have indicated that genetic recombination events frequently occurred among HBoV strains. Recent studies demonstrated the possibility that NS1/NP1 gene of HBoV-3 was derived from HBoV-1 whereas VP1/2 originated from HBoV-2 as well as HBoV-4 that was derived from recombination of NS1/NP1 of HBoV-2 and VP1/2 of HBoV-3 (Kapoor et al., 2010; Khamrin et al., 2013). Moreover, HBoV-3 showed evidence of genetic recombination with HBoV-1 and HBoV-4 (Cheng et al., 2011; Chieochansin et al., 2010; Fu et al., 2011). Evidence of genetic recombination located at VP1/2 gene of CBoV TH-2016 as identified in this study should be considered for further study.

Recent studies have indicated that the unique deletion in VP1/2 of CBoV-2, the absence of ORF4, or both, are associated with viral virulence. Also we speculate about the possible association of a longer NS1 with viral virulence. However, since we cannot determine whether CBoV TH-2016 has indeed replicated in the respiratory tract, further conclusions about viral pathogenesis and virulence cannot be made from our data. As clinical manifestations and genetic organization of animal and human bocaviruses appear to be quite similar, further studies of natural and experimental infections of bocaviruses in dogs may shed light on the molecular basis of the pathogenesis and virulence of members of the genus *Bocaparvovirus*.

Canine circovirus, CanineCV, a novel non-porcine circovirus was firstly established and associated with vasculitis and hemorrhagic syndrome (Li et al., 2013). Several studies also detected the CanineCV genome from intestinal samples and attempted to find the association of the CanineCV with intestinal disease (Anderson et al., 2017; Decaro et al., 2014; Hsu et al., 2016; Li et al., 2013; Thaiwong et al., 2016).

However, clinical association and pathogenic role of CanineCV infection are still undetermined. In this study, we discovered CanineCV genome from two dogs' lungs (14P105D, 14P112N) suffering from respiratory distress by using NGS.

Analysis of nearly complete genome of CanineCV detected in Thailand showed that they were clustered into clade that closely related to CanineCV-UCD3-478 strain, which circovirus was detected in serum of American dog. Surprisingly, pairwise distance analysis of CanineCV Thai isolates showed at least 13.4% genetic difference from closed-related strain even though they felt into the same clade, suggesting novel strains of CanineCV were discovered in this study. Furthermore, potential naturally genetic recombination events were reported in these CanineCV strains, predicted the recombination breakpoint occurring at Rep gene, which is overlapping the ORF3 region. According to *Circoviridae* family, the recombination of beak and feather virus (Heath et al., 2004) and Torque teno virus (Manni et al., 2002) and in many strains of PCV2 (Hesse et al., 2008; Ma et al., 2007) were reported. These findings suggested the possibility of natural recombination events occurring in other circoviruses.

The ORF3, a recently recognized overlapping anti-directional region of ORF1 gene, has been related to the normal responsibility for virus-induced apoptosis in PCV2 infection (Liu et al., 2005) and the dispensable region for virus replication (Juhan et al., 2010a); the ORF3 was also found in this novel CanineCV TH-2016 strains. Interestingly, recent studies showed that ORF3-deficient PCV2-infected mice showed less severity than those infected with wild type strains (Liu et al., 2006); however the evidence of pathogenicity of ORF3-deficit PCV2-infected pigs was limited (Juhan et al., 2010a). Consequently, function of the ORF3 region as well as an altering mutation in this region of any circovirus should be taken into account. Even though the ORF3 region of CanineCV genome has been described, but it did not gain any attention (Li et al., 2013). Interestingly, the recombination breakpoint of CanineCV strains occurred in Rep gene, which is intersected with the ORF3 region, was found in Thai isolates. This finding shed a light on the possibility of recombination that might alter the pathogenicity and virulence of CanineCV.

CanineCV genomic data obtained from this study contributes to a novel insight and a better knowledge of the canine circovirus in a regional context showing the existence of novel emergence of recombinant CanineCV strains reported in Thailand. Further study should be focused on the function of each region of CanineCV genome, which might contribute better understanding.

Canine infectious respiratory disease complex is a complex disease caused by multifactorial etiologies. The one important factor involved with this complex is infectious pathogens. In this present study, we emphasized to develop the multiplex PCR assays in order to investigate the CIRDC associated viruses circulating in Thailand. These techniques yielded effective results and could be used as screening methods for disease surveillance. The assays also are user-friendly, non-consumable and rapid processes. A massive epidemiology of CIRDC associated viruses was surveyed among 2 different populations, resulted to differentiate the possible associated risk factors for CIRDC-virus infection. The epidemiology of CIRDC virus infection in Thailand was established and gained for further disease quarantine and control. Moreover, novel canine bocavirus and canine circovirus strains were firstly identified in this study by using next generation sequencing method. Genomic analysis of both viruses suggested evidence of genetic recombination occurred among viral strains derived from various countries. This study also shed novel insights for diseases and novel virus distributions in Thailand as a first report. Further study should be conducted to investigate the novel viruses for further disease monitoring, control and eradication.

3.2 LIMITATION OF THE STUDY

1. Study samples were collected from dogs locating in Thailand that were being subjective because of number of samples, geographic distributions, clinical appearances and duration of the study.

2. This study was challenged with statistical analysis and interpretation based on varying outputs from un-collected data.

3. Only negative-CIRDC-mPCR samples were subjected to detect other possible causing pathogens by NGS, resulting in missing data of discovery of novel pathogens in the positive-mPCR samples.

3.3 SUGGESTION FROM THE STUDY

1. Simultaneous multiplex PCR assays could be used as screening methods to detect a common respiratory virus associated respiratory disease in dogs effectively.

2. Further surveillance of the common respiratory viruses and possible novel pathogen should be investigated, yielding epidemiological data for further information.

3. Novel canine bocavirus 2 and canine circovirus were firstly established in this study, suggesting the viruses were circulating in Thailand and gained possible etiology for respiratory pathogens in dogs.

4. Pathogenicity of the canine bocavirus 2 and canine circovirus should be taken into account for novel insight in pathogenesis.

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APPENDIX

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PUBLICATIONS

1. Piewbang C., Rungsipipat A., Poovorawan Y., Techangamsuwan S. 2016. Development and application of multiplex PCR assays for detection of virus-induced respiratory disease complex in dogs. J Vet Med Sci. 78(12), 1847-1854.

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5. Piewbang C. and Techangamsuwan S. Canine infectious respiratory disease complex viruses: a diagnostic platform, status and discovery. 2016. Thai J Vet Med 46: 89-90.

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1. Piewbang C., Rungsipipat A., Poovorawan Y., Techangamsuwan S. Cross- sectional investigation and risk factor analysis of community-acquired and hospital-associated canine infectious respiratory disease complex. Plos One. (Submitted)

2. Piewbang C.,Jo WK., Puff C., Ludlow M., van der Vries E., Banlunara W., Rungsipipat A., Kruppa J., Jung K., Techangamsuwan S., Baumgärtner W., Osterhaus A.D.M.E. 2017. A novel strain of canine bocavirus type-2 infection associated with intestinal lesions reminiscent of canine bocavirus type-1 infection. Vet Pathol (Accept with revision)

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3. Piewbang C., Rungsipipat A., Lombardini ED., Lacharoje S. and Techangamsuwan S. Immunophenotyping of cutaneous extramedullary plasmacytoma in two dogs. Poster and proceeding of the 6th Asian Society Veterinary Pathology Conference and 5th Malaysian Association of Veterinary Pathology, November 22-24, 2013 Malaysia

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5. Piewbang C., Rungsipipat A. and Techangamsuwan. S. Fatal systemic canine herpesvirus infection associated with acute death in neonatal puppies. Oral presentation and proceeding of the 14th Chulalongkorn University Veterinary Congress, April 20-22, 2015 Bangkok, Thailand

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