รายงานวิจัยฉบับสมบูรณ์

โครงการ การพัฒนาปฏิกิริยาลูกโซ่โพลีเมอเรสแบบมัลติเพล็กซ์และการวิเคราะห์เมตาจีโนมิค ในโรคระบบทางเดินหายใจสุนัขแบบซับซ้อนที่เกิดเนื่องจากไวรัส

(Development of multiplex RT-PCR and metagenomic analysis of canine infectious respiratory disease complex associated viruses in dogs)

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บทคัดย่อ

โรคติดเชื้อทางระบบทางเดินหายใจในสุนัขนับว่าเป็นโรคที่มีความซับซ้อนที่เกิดได้จากหลายสาเหตุ เชื้อไวรัสนับว่ามีบทบาทสำคัญในการก่อโรคและการดำเนินไปของโรค ซึ่งยังไม่มีการศึกษาอย่างแท้จริงในประเทศไทย การศึกษาในครั้งนี้จึงได้พัฒนาวิธีการตรวจวินิจฉัยด้วยเทคนิคมัลติเพล็กซ์พีซีอาร์เพื่อใช้ในการสำรวจโรค โดยเป็นวิธีที่มีประสิทธิภาพ สามารถใช้ในการตรวจคัดกรองโรคเบื้องต้นได้อย่างรวดเร็วเมื่อเทียบกับเทคนิคพีซีอาร์แบบทำทีละปฏิกิริยาหรือชุดตรวจสำเร็จรูปข้างตัวสัตว์ ทำการเก็บตัวอย่างจากเยื่อบุช่องจมูกและช่องคอหอยส่วนปากในสุนัขที่แสดงอาการป่วยทางระบบทางเดินหายใจ เพื่อตรวจหาเชื้อไวรัสก่อโรคระบบทางเดินหายใจซับซ้อนในสุนัขด้วยเทคนิคมัลติเพล็กซ์พีซีอาร์ และศึกษาทางด้านระบาดวิทยาของเชื้อไวรัสก่อโรคที่พบบ่อย ได้แก่ ไวรัสอินฟลูเอนซา ไวรัสพาราอินฟลูเอนซา ไวรัสไข้หัดสุนัข ไวรัสโคโรนาในระบบทางเดินหายใจ ไวรัสอะดีโนชนิดที่สอง และไวรัสเฮอร์ปีส์ชนิดที่หนึ่ง นอกจากนี้ยังได้ศึกษาถึงปัจจัยเสี่ยงต่างๆ ความเกี่ยวข้องของระดับความรุนแรงของอาการแสดงกับแหล่งที่อาจติดโรคและจำนวนเชื้อไวรัสที่ตรวจพบ ผลการศึกษาในครั้งนี้ยังค้นพบเชื้อไวรัสใหม่ 2 สายพันธุ์ ได้แก่ ไวรัสโบคาชนิดที่สอง และไวรัสเซอร์โค ซึ่งแยกได้จากสุนัขที่มีอาการทางระบบทางเดินหายใจแต่ให้ผลลบทั้งหมดกับเชื้อไวรัสที่พบบ่อยข้างต้นเมื่อตรวจด้วยเทคนิคมัลติเพล็กซ์พีซีอาร์ การค้นพบอาศัยเทคโนโลยีขั้นสูงในการถอดรหัสพันธุกรรมและการวิเคราะห์เมตาจีโนมิค สำหรับไวรัสโบคาสายพันธุ์ที่ค้นพบนี้ (CBoV TH-2016) มีรหัสพันธุกรรมที่แตกต่างจากที่มีรายงานในประเทศฮ่องกงและเกาหลีใต้ ในขณะที่ไวรัสเซอร์โค (CanineCV TH-2016) พบการผสมกันระหว่างสายพันธุ์ที่มีต้นกำเนิดจากประเทศสหรัฐอเมริกาและจากประเทศจีน หลังจากการวิเคราะห์ความเหมือนกันของลำดับนิวคลีโอไทด์พบว่าไวรัสที่พบใหม่ทั้ง 2 สายพันธุ์มีลักษณะจำเพาะ และสามารถจำแนกได้ว่าเป็นสายพันธุ์ใหม่ โดยสรุป การศึกษาในครั้งนี้ได้เน้นถึงการตรวจหาไวรัสก่อโรคในระบบทางเดินหายใจในสุนัขได้สำเร็จ และยังค้นพบเชื้อไวรัสใหม่ที่ไม่เคยมีรายงานมาก่อนในประเทศไทย ซึ่งจะช่วยยกระดับการเฝ้าระวังโรคและการวินิจฉัยโรคให้กับสัตวแพทย์ได้

คำสำคัญ โรคติดเชื้อทางระบบทางเดินหายใจในสุนัข ไวรัสอุบัติใหม่ ระบาดวิทยา เมตาจีโนมิค
มัลติเพล็กซ์พีซีอาร์

Abstract

Canine infectious respiratory disease (CIRD) is a complex disease caused by multifactorial etiologies. Infectious pathogens, particularly viruses, play an important role for the disease progression that is not fully documented yet in Thailand. In this present study, the developed diagnostic modules, multiplex polymerase chain reaction (mPCR), were established for disease surveillance, yielding simultaneously effective method for disease screening when compared with single PCR assay and rapid immunogenic test kits. Furthermore, both nasal and oropharyngeal samples were prospectively collected from respiratory ill dogs residing in Thailand, for CIRDC associated virus detection using developed mPCR assays. Epidemiology of viral associated respiratory disease complex was achieved and has been suggested that all the common CIRDC-viruses including 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 type 1 (CaHV-1), have been circulating in Thailand. In addition, risk factors of infections, severity of clinical presentations among different possible sources of infections and numbers of detected viruses were investigated. Interestingly, two novel virus strains, canine bocavirus type 2 (CBoV-2) and canine circovirus (CanineCV), were discovered from PCR-negative CIRDC respiratory suffering dogs by applying next generation sequencing (NGS) and metagenomics analysis. For CBoV-2 isolated from this study, named CBoV TH-2016 strain, showed different sequences from previously described CBoV-2 strain isolated from Hong Kong and South Korea. Concordantly, the phylogenetic analysis of our CanineCV TH-2016 isolates presented the recombinant strains among USA- and China-derived CanineCV strains. After pairwise identity matrix analysis, it revealed that both viruses were unique and subjected to novel strains. In conclusion, this study emphasized the viruses associated with respiratory problems in dogs and are currently circulating in Thailand. The discovered novel viruses also raised awareness of veterinarian for disease monitoring and diagnosis.

Keywords: Canine infectious respiratory disease, Emerging virus, Epidemiology, Metagenomics, Multiplex PCR

Executive Summary

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. 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 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 nasal (NS) or oropharyngeal (OS) swabs.

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. 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 were 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. 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. 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.

Development of multiplex RT-PCR and metagenomic analysis of canine infectious respiratory disease complex associated viruses in dogs

I. Introduction

Canine infectious respiratory disease (CIRD) is a disease complex occurring in dogs, affecting the larynx, trachea, bronchi, and occasionally the nasal mucosa (Appel and Binn, 1987). 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 just dealing with germs, but also environmental factors and host immune responses are playing an equally important role in this disease complex (Erles et al., 2004). The pathogens causing CIRDC are consisted either viruses, bacteria or both, which transmit by aerosol from infected dogs particularly living in poor ventilation kennels, animal shelters and veterinary hospitals (Appel and Binn, 1987). The clinical manifestation is varied 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 studies regarding 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) (Binn et al., 1967; Ditchfield et al., 1962; Erles et al., 2004; Posuwan et al., 2010; Wright et al., 1972), canine distemper virus (CDV) (Posuwan et al. 2010; Pratakpiriya et al., 2017; Radtanakatikanon et al., 2013), canine herpes virus (CaHV-1) (Buonavoglia and Martella, 2007; Erles et al., 2004; Karpas et al., 1968), canine influenza virus (CIV) (Buonavoglia and Martella, 2007; Payungporn et al., 2008; Posuwan et al., 2010) and canine respiratory coronavirus (CRCoV) (Buonavavoglia and Martella, 2007; Decaro et al., 2007; Ellis et al., 2005; Erles and Brownlie, 2005; Erles et al., 2003).

Virus properties

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), hemagglutin-neuraminidase (HN) and large (L) genes. The HN protein is involved in cell attachment to initiate virus infection. The F protein can mediate fusion of viral envelope with cell membrane (Buonavoglia and Martella, 2007). The CPIV mostly replicates in cells in 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 (Appel and Binn, 1987; Binn et al., 1979). In addition, the virus also promotes *Bordetella bronchiseptica* infection in respiratory tract (Binn et al., 1979; Ueland, 1990). CPIV infected dogs usually show catarrhal rhinitis and tracheitis with mononuclear and polymorphonuclear inflammatory cells infiltration into mucosa and submucosa (Appel and Percy, 1970). The CPIV is found frequently at the upper respiratory tract of infected dogs, but it can sporadically be isolated from lungs, spleen, kidneys indicating the possibility of systemic infection (Ford and Vaden, 1998). Simultaneous infection with other viruses or secondary bacterial attack such as *Bordetella bronchiseptica* always promote worse clinical symptom (Binn et al., 1979; Ueland, 1990). The parainfluenza virus also causes respiratory disease in mammals and birds (Buonavoglia and Martella, 2007). Moreover, CPIV 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 (Chatziandreou et al., 2004; Randall et al., 1987).

Canine adenovirus type 2 (CAdV-2)

Canine adenovirus (CAdV), a double stranded DNA virus, is belonged to *Adenoviridae* family. CAdV is subdivided in to two types; type 1 and type 2 (Buonavoglia and Martella, 2007). The target cells of CAdV among types are different. 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 is route of infection for CAdV-2. The virus replicates in respiratory epithelium and mucosal epithelium of pharynx as well as tonsillar crypts and pneumocyte type II cells (Appel and Binn, 1987; Curtis et al., 1978). Therefore, CAdV-2 has been more commonly associated with the CIRDC. The infection usually occurs in puppies but sporadically detected in adult dogs (Buonavoglia and Martella, 2007). Dogs exposed to CAdV-2 alone rarely show spontaneous clinical signs. Bronchiolitis obliterans and interstitial pneumonia will be extensive, if the CAdV-2 dogs are involved with additional other pathogens, leading to complex respiratory infection (Appel, 1987;

Appel and Binn, 1987; Curtis et al., 1978; Koptopoulos and Cornwell, 1981). Moreover, the CAdV-2 can produces lesion in gastrointestinal tract due to its tissue tropism and causes mild degree of diarrhea (Swango et al., 1970). The host range of the virus is broaded 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; Skilling 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)

Canine distemper virus (CDV), a single-stranded negative-sense RNA virus, belongs to Paramyxoviridae family. The virus composes of six proteins gene; 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. 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 cell host, fusogenic activity and may also play a role in host's humoral immune response. So, the F and H proteins are the most variable parts of virus and have often been used to assess genetic changes between CDV isolates (Lee et al., 2010; Pardo et al., 2005; Radtanakatikanon et al., 2013). Lymphocytes and epithelial cells serve as a cell tropism of CDV after dogs are transmitted by aerosol (Carvalho et al., 2012; Pratakpiriya et al., 2017). Therefore, respiratory tract, gastrointestinal tract, integumentary system, lymphatic system, urinary bladder and central nervous system are affected to CDV infection (Carvalho et al., 2012; Erles et al., 2004; Radtanakatikanon et al., 2013). As CDV is one of the most fatal CIRDC viruses; it is frequently associated with severe respiratory symptoms and accompanied by systemic lesions such as gastrointestinal, integumentary, and central nervous systems. Lymphoid tissues and epithelial cells are main targets of CDV replication causing lymphoid depletion and immunosuppression (Carvalho et al., 2012). At final stage of infection, CDV causes encephalitis and demyelination when the virus infects the central nervous system of dogs. 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 herpesvirus (CaHV-1)

Canine herpes virus (CaHV-1) is an alphaherpes virus, which is a group 1 double stranded DNA virus. The viral genome contains 125 Kbp comprising of a unique long (97.5 Kbp) and short 7.7 Kbp and flanking by terminal and internal inverted repeats (Papageorgiou et al., 2016). The CaHV-1 has been isolated from dogs with CIRDC, but its role remains uncertain (Binn et al., 1979; Erles et al., 2004; Karpas et al., 1968). The CaHV-1 infected puppies show fatal generalized necrotizing and hemorrhagic lesions (Piewbang et al., 2017). On the contrary, infection in older dogs mostly appears to be restricted to upper respiratory tract, which shows mild clinical symptoms of rhinitis and pharyngitis (Carmichael et al., 1965). However, the CaHV-1 can pass through fetal membranes from infected dam, resulting to fatal death (Hashimoto et al., 1982). 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 (Miyoshi et al., 1999; Okuda et al., 1993).

Canine influenza virus (CIV)

Canine influenza virus is influenza A virus, 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 formed 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). 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; Payungporn et al., 2008; Posuwan et al., 2010; Yoon et al., 2005). 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 genetically different from canine enteric coronavirus and is classified to coronavirus group 2 as same as bovine coronavirus and human coronavirus OC43. This finding suggests a recent common ancestor of these viruses and demonstrates the occurrence of repeated host-species shifts (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 United Kingdom (Priestnall et al., 2006), Japan (Yachi and Mochizuki, 2006), Italy (Decaro et al., 2007), and Korea (An et al., 2010). 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). 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 superinfection and colonization of other pathogens (Buonavoglia and Martella, 2007).

Diagnostic modules

Diagnosis of CIRDC virus becomes important for appropriate treatment plan, prognosis and prevention strategies. Various tests are available to diagnose these infections; however, many are not practical due to long processing time, poor specificity or sensitivity, and costly diagnostic tools (Porsuwan et al., 2010). Some of these methods include viral culture and isolation technique. Such modalities cannot perform in all viruses due to poor ability of virus growth in cell culture system. 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 appropriate method to detect these viruses. Multiplex Polymerase Chain Reaction (mPCR) technique has been used to diagnose multiple pathogens (Payungporn et al., 2006; Thontirawong et al., 2007). On the other hand, 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 (Shigayeva et al., 2007), 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 zoonoses potential. Moreover, some viruses may increasingly turn virulence of infectivity to infect the 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 (Fakruddin et al., 2012; Ronaghi, 2001). 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 to known viruses to cross-react. 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 pyrosequencing has 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 question. Alternatively, most viruses that cause CIRDC in dogs are genetically similar to viruses that cause respiratory disease in humans as well.

II. Objectives

1. 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

2. To establish disease surveillance each particular virus for epidemiological monitoring the potentially air-borne zoonoses from animals to humans

3. 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

4. To discover the novel strain of virus or novel virus that can be or not associated with respiratory illness in dogs.

5. To monitor the emerging virus which is possible to transmit to other animals and humans, alerting to public health concerns.

III. Materials and Methods

Part I. Development of multiplex RT-PCR, cross-sectional study and risk factor analysis of canine infectious respiratory disease complex associated viruses in dogs

1. Study design and population, clinical information, sample collection and positive control preparation

1.1) 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).

1.2) Study population

Two hundred and nine dogs with respiratory illness were included in the study, taking a nasal swab (NS) and an oropharyngeal swab (OS) from each dog. These 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 "community-acquired infection (CAI)" and "hospital-associated infection (HAI)" dog groups. The CAI group defined as 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. Moreover, respiratory suffering dogs, which were subjected for post mortem examination, were also randomly collected various organs including lung, liver, kidneys and intestines for further investigation.

1.3) Clinical information

Clinical signs, including nasal discharge, coughing, dyspnea and presence of pneumonia, were respectively recorded by a veterinarian on the date of sampling. The clinical signs were then scored by "1" if dogs had either nasal discharge or cough, "2" if dogs had nasal discharge and cough, "3" if dog had nasal discharge or/ and cough with dyspnea and "4" if dogs presented an evidence of bronchopneumonia by physical examination or radiographic diagnosis. The scores was further graded for the clinical severity 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.

1.4) Sample collection

The NS samples were collected by gentle inserting a sterile rayon tipped applicator (Puritan®, USA) into the nostril to a depth of approximately 2 cm and rolling the swab onto the nasal planum. The OS samples were collected by likewise swabbing on the tonsil. The swabs were then immersed in 0.5 ml of 1% sterile phosphate buffered saline (PBS) and stored at -80°C until assayed. The fresh tissues from necropsied dogs with respiratory distress were sterile collected and stored at -80°C for further investigation.

1.5) Positive control preparation

Positive controls for CDV, CPIV, CAdV-2 were obtained from modified-live vaccine Vanguard® plus 5/CV-L (Zoetis, Michigan, USA), containing CPIV ($10^{5.0}$ $TCID_{50}$/ ml), CDV ($10^{2.5}$ $TCID_{50}$/ ml) and CAdV-2 ($10^{2.9}$ $TCID_{50}$/ ml). The positive controls for CRCoV, CaHV-1 and CIV

were obtained from naturally infected dogs that were confirmed by nucleic acid sequencing. The H3N2 CIV positive control was kindly provided by Professor Dr. Alongkorn Amornsin, Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University. Each CIRDC RNA and DNA virus-positive controls were then prepared for multiplex RT-PCR and multiplex PCR developments.

2. Nucleic acid extraction and cDNA synthesis

2.1) Nucleic acid extraction and quantification

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).

2.2) cDNA synthesis

The RT was performed using 100 ng RNA as the template for complementary DNA (cDNA) synthesis by the Omniscript® Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany). To improve the cDNA quality, the RNA was extended by pre-heating at 65 °C for 5 minutes. Then the master mixes of solution were added to the reaction tube including 0.5 μM of each dNTP, 1 μM of oligo-dT primer, 2 μl of 10x Buffer RT, 10 units of RNase inhibitor and 4 units of reverse transcriptase enzyme with a final volume of 20 μl. The mixtures were incubated at 25°C for 10 minutes, followed by 37°C for 60 minutes then 93°C for 5 minutes. The cDNA and DNA were stored at -20°C until used for further PCR amplifications.

3. Specific primer designs

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 et al., 2004; Decaro et al., 2010; Payungporn et al., 2008; 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 re-aligned 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. Moreover, the canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as internal control (Techangamsuwan et al., 2009).

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 (First round PCR)
	CoV_16594_R	TAYTATCARAAYAATGTCTTTATGTC		
	CoV_Pan_16510_R	TGATGATGGNGTTGTBTGYTATAA		458 (Second round 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 B

4. Optimization of PCR assays for CIRDC detection

4.1) Optimization of simplex PCR

Prior to performing the PCR for detection of RNA viruses, a first round nested 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 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The final extension was performed at 72 °C for 7 min. Subsequently, the amplified CRCoV product of the first nested 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, excepted for the gradient annealing temperature (Ta) was programmed ranging from 50°C to 59°C in order to optimize the reaction. Thermal cycling was performed with 95 °C for 5 min, then 40 cycles of 95 °C for 1 min, varied Ta for 1 min and 72 °C for 1 min, and then finally 72 °C 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 transilluminator and compared to expected size of the PCR product (Table 1).

4.2) 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 compositions were as 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.

5. Nucleic acid purification, sequencing and phylogenetic analysis.

PCR amplicons was sequenced 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.

6. Specificity and sensitivity test

6.1) Specificity test

Specificity of the each PCR assay was evaluated by cross-reaction tests, performing on nucleic extracts from isolates viruses, which is belonging to CIRDC viruses except the virus that specific to that PCR test. Moreover, some other viruses that belong to same family; however, it is either pathogens or not pathogens for dogs was used for specificity test such as Newcastle disease virus (NDV), canine parvovirus (CPV) and feline herpes virus (FHV).

6.2) Sensitivity test

To access the analytical sensitivity of each simplex PCR assay, Concentration of nucleic acid was calculated by using Nanodrop spectrophotometer at 260 nm. two-fold serial dilutions of nucleic extracted positive controls were amplified. The ten dilutions of tested controls were 2^{0}–2^{-10} ng/ PCR reaction.

7. Statistical analysis

Descriptive analysis was used as the median for age. Data were evaluated using the SPSS statistical analyser version 22.0 software (IBM Corp, New York, USA). Differentiation of the CAI and HAI groups was compared using Fisher's exact and χ^2 tests. Clinical severity against the source of infection and age 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 infection were 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.

Part II. Metagenomic analysis of canine infectious respiratory disease complex associated viruses in dogs.

1. Study design and sample collection

Negative CIRDC virus detection samples by using the developed multiplex PCRs were subjected to metagenomic analysis using next generation sequencing (NGS) technology. Selective lungs samples from respiratory suffering dogs were included for metagenomic study.

2. Sequence independent and single primer amplification and next generation sequencing

Lung samples of selected dogs were processed for sequence independent single primer amplification (SISPA). Briefly, the lungs were individually homogenized under aseptic conditions. The supernatant was collected as processed to extract the nucleic acid using Qiamp Viral RNA mini kit (Qiagen, Germany) following manufacturer's recommendation. The extracted RNA was then processed to cDNA by reverse transcription using SuperScript IV First Strand Synthesis (Invitrogen, Thermo Fisher Scientific, USA) and klenow fragment amplification following manufacturer's protocol. The cDNA was later subjected to random PCR amplification using Taq polymerase. The PCR product was purified by Monarch® PCR and DNA clean up kit (New England Biolab, UK) and then run on 1% agarose gel with Gel Red in-gel staining in order to prove the quality of Taq amplification. PicoGreen (Invitrogen, Thermo Fisher Scientific, USA) was used to measure final DNA concentration. A DNA Library was then constructed following Nextera XT protocol (Illumina, USA). Samples were then deep sequenced on an Illumina MiSeq system using MiSeq Reagent kit V3 (300x2 cycles).

3. Next generation sequencing analysis

Raw reads outputs were trimmed and assembled, later 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. Interesting sequences were blasted with references sequences available in GenBank by using Blastn tools.

4. PCR specific targeting interesting genomes and whole genome sequencing

In order to complete nearly full-length novel genome, the specific primers were designed based on sequence obtaining from NGS reads. PCR reactions were comprised of a mixture of 1U of Phusion DNA polymerase (New England Biolab, UK), 10 mM of dNTP in 5x Phusion HF Buffer, 10 µM final concentration of each primer, 2 µl of template, and made up to 50 µl with nuclease-free water. All specific PCR were performed as the same cycler conditions; initial denaturation for 98ºC for 30 sec, followed by 45 cycles of 98ºC, 20 sec, 50ºC, 30 sec, 72ºC, 1 min and 72ºC, 7 min for final extension. The PCR product was run in 1% agarose gel. The gel containing positive PCR products were purified by using Monarch DNA Gel extraction kit (New England Biolab, UK). The various virus strains which were concordantly to the NGS results and available in GenBank, were obtained and aligned with the NGS reads. The sequences were multiple aligned using MAFFT alignment version 7 (http://mafft.cbrc.jp/alignment/server/) and MEGA 7 based on nucleotide and deduced amino acid sequences of near full-length genome representing in GenBank. Phylogenetic tree was constructed using neighbor-joining method with GTR+G+I model. Bootstrap analysis was performed using 1000 replicates. Pairwise distance of novel genome and other interesting genes were calculated using BioEdit. Less than 85% nucleotide identity and deduced amino acids comparing with genome database in GenBank were classified as a novel virus.

IV. Results

1. Animal data from epidemiology investigation

The 209 dogs from four regions of Thailand including Central (n=127), Eastern (n=21), Southern (n=49) and Western (n=12) were categorized into 133 CAI dogs and 76 HAI dogs based on the presentation of their respiratory symptoms and history of hospital visits. Vaccinations were performed using the commercially combined CPIV-CDV-CAdV-2 vaccine and accounted for 32.3% (43/133) and 28.9% (22/76) dogs in the CA and HA 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. Other presented clinical signs are shown in Table 2 and noted that 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 group dogs.

Table 2 Clinical signs of respiratory ill dogs in the community-acquired infection (CAI) and hospital-associated infection (HAI) groups

Study population	Percentage of presented clinical signs (n)			
	Nasal discharge	Cough	Dyspnea	Pneumonia
CAI	78.2 (104/133)	66.2 (88/133)	49.6 (66/133)	28.6 (38/133)
HAI	78.9 (60/76)	61.8 (47/76)	53.9 (41/76)	30.3 (23/76)

2. Development of multiplex PCRs for CIRDC detections

Multiplex RT-PCR and multiplex PCR to detect CIRDC-RNA and –DNA viruses were optimized by performing on 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. 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 and 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).

In order to validate the developed PCR in practice, 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 3). 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 positive predictive value (PPV; 100%) of all multiplex PCRs was consistent with the specificity (100%), while the negative predictive value (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.

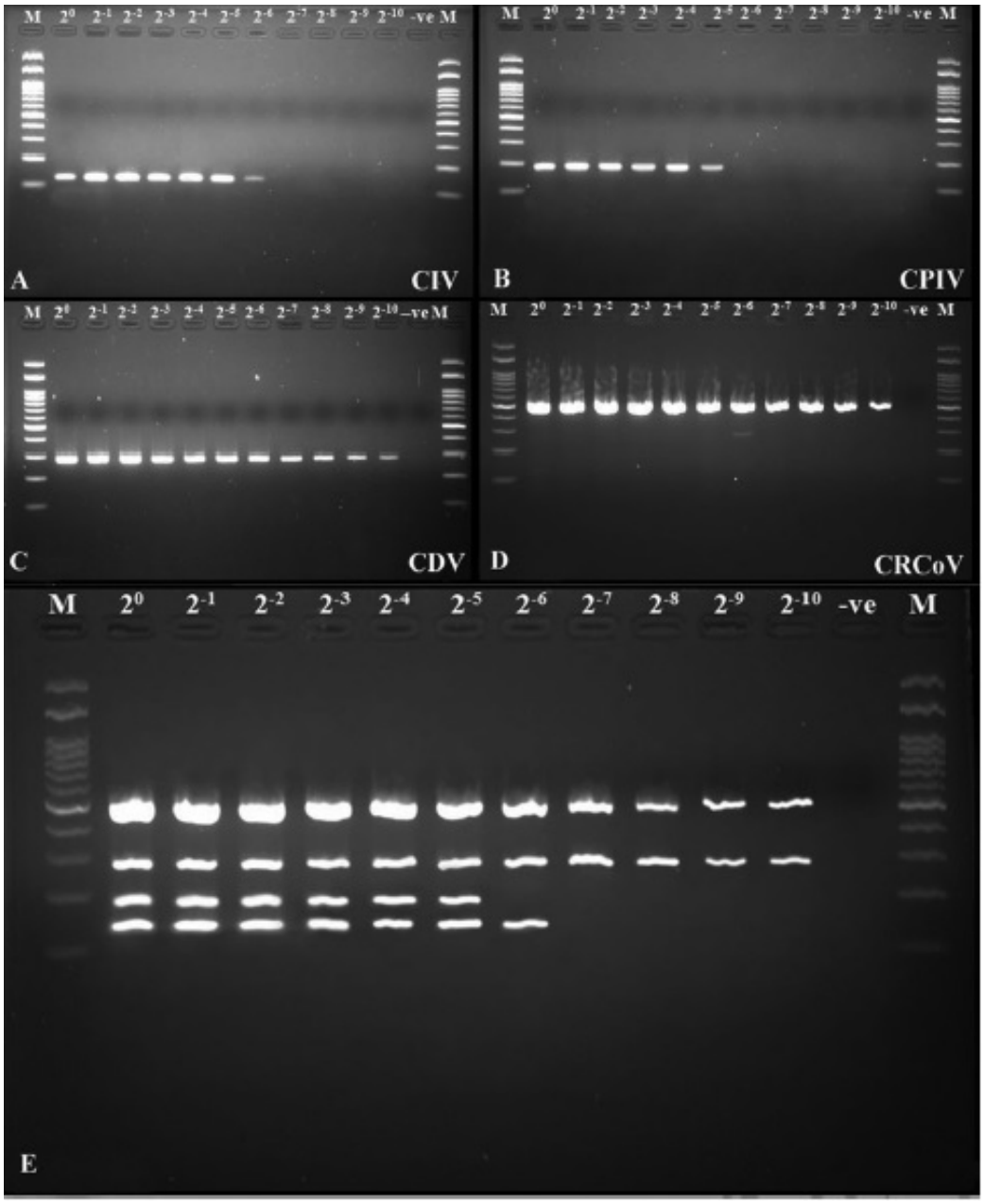

Fig. 1 Analytical sensitivity test of the (A–D) simplex and (E) multiplex RT-PCR of RNA-associated CIRDC viruses. (A) CIV, (B) CPIV, (C) CDV and (D) CRCoV. Two-fold serial dilutions of the positive controls ranging from 2^{0} –2^{-10} ng/reaction were assayed. Detection threshold was equal in both the 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.

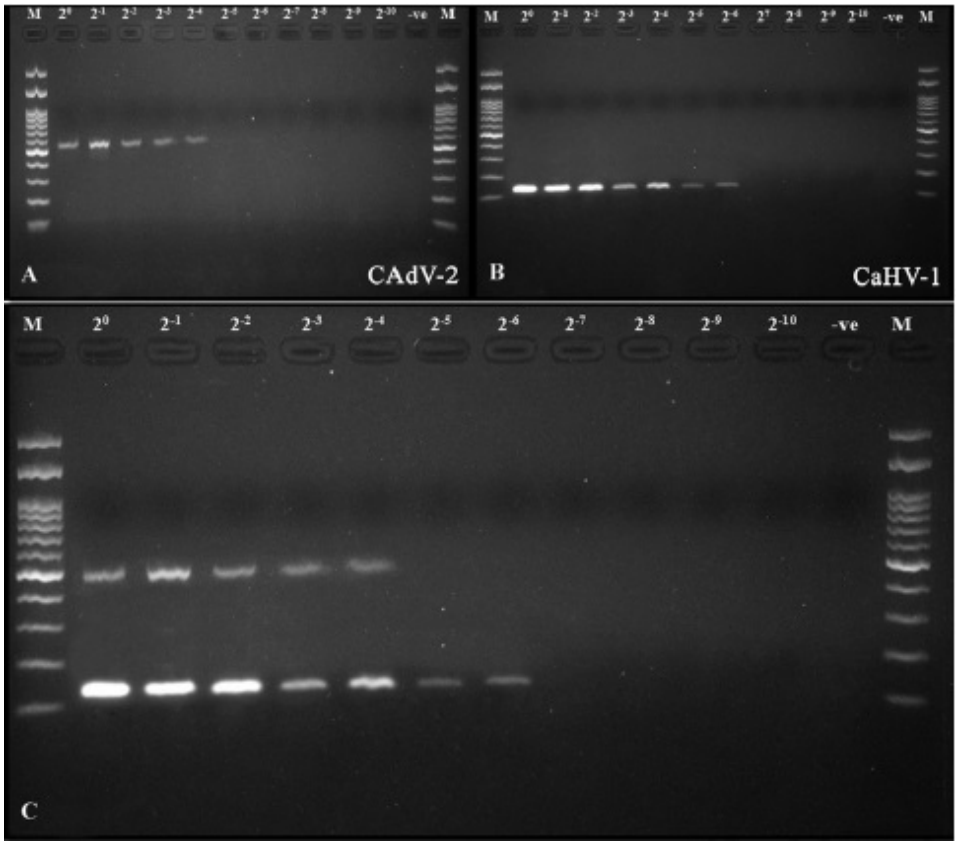

Fig. 2 Analytical sensitivity test of (A, B) simplex and (C) multiplex PCR of DNA-associated CIRDC viruses. (A) CAdV-2 and (B) CaHV-1. Two-fold serial dilutions from 2^{0} –2^{-10} ng/reaction were tested. Detection threshold was similar in both the simplex and multiplex modalities and revealed minimal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

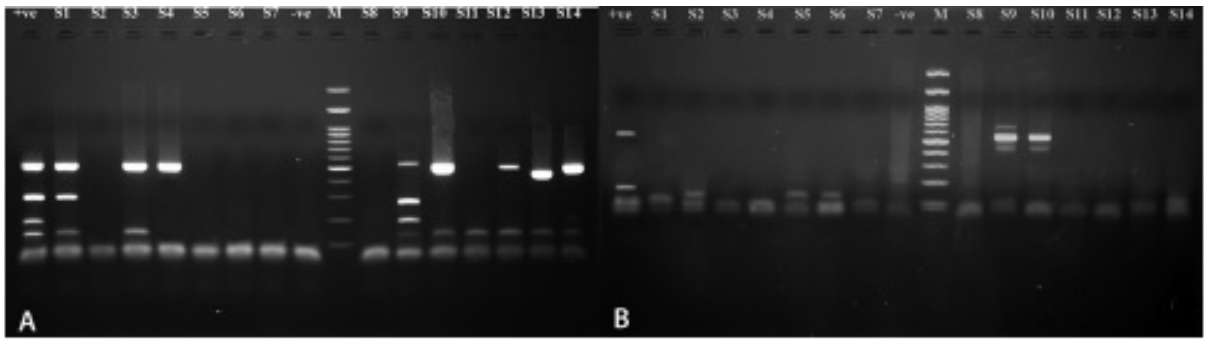

Fig. 3 Results of the (A) multiplex RT-PCR and (B) multiplex PCR tested on clinical samples (S1–S14). M=DNA marker 100 bp, –ve= negative control, +ve= positive control.

Table 3 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 3 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

3. Prevalence of CIRDC viruses in Thailand during 2013-2015 and risk factor analysis

The prevalence of CIRDC viruses was analyzed by multiplex PCR assays, which 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 (57.0–62.0%), while CAdV-2 was the least detected (8.3–11.8%). The other infections in CAI group were CDV and then CPIV and CaHV-1, respectively, while in the HAI group it was the same viruses but in the order of CPIV, CaHV-1 and CDV. The CAI dogs had a significantly higher prevalence of CDV infection than the HAI dogs (P = 0.0049; Table 4).

Table 4 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/total number assayed) and (below) the 95% confidence limits *Statistically significant

For multiple virus infections, the prevalence was 81.2% (108/133) in CAI dogs and 78.9% (60/76) in HAI dogs (Table 5).

Table 5 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)	43.4 (33/76)	0.248
	27.72-43.78	32.86–54.62	
3 viruses	30.1 (40/133)	27.6 (21/76)	0.709
	22.91–38.86	18.78–38.63	
4 viruses	13.5 (18/133)	5.3 (4/76)	0.061
	8.65–20.47	1.67–13.16	
5 viruses	2.3 (3/133)	2.6% (2/76)	0.864
	0.48–6.72	0.17–9.65	
6 viruses	0	0	-

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

The increased number of viral co-infections was similar in both groups and revealed that double viral infections were most frequently found. The frequency of multiple infections in the two groups decreased with increasing numbers of viruses, with no multiple infections of all six virus types being found in either the CAI or HAI dogs. There was no significant difference between the CAI and HAI dogs for multiple infections.

When the variable demographic factors and single or multiple CIRDC virus infections 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 of infection, vaccination status and respiratory signs against single or multiple CIRDC-virus infections (Table 6). 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 7). The respiratory score was compared with CIRD agents detected in this study in both CAI and HAI (Table 8). 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 infection of CIV and CRCoV was also predominately detected in both groups with statistical association (P = 0.009, Table 8).

Table 6 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]	

Table 6 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]	
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 7 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]	

Table 7 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]	
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]	
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 8 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
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

Table 8 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		
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 coinfections)

The weather in Thailand was examined for any associated risk of CIRCD incidence in the period between March 2013 and April 2015. The average humidity, temperature and amount of rain fall were compared with the number of CIRDC infected dogs (Fig. 4). 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 rain fall (range 190.6–245.9 mm).

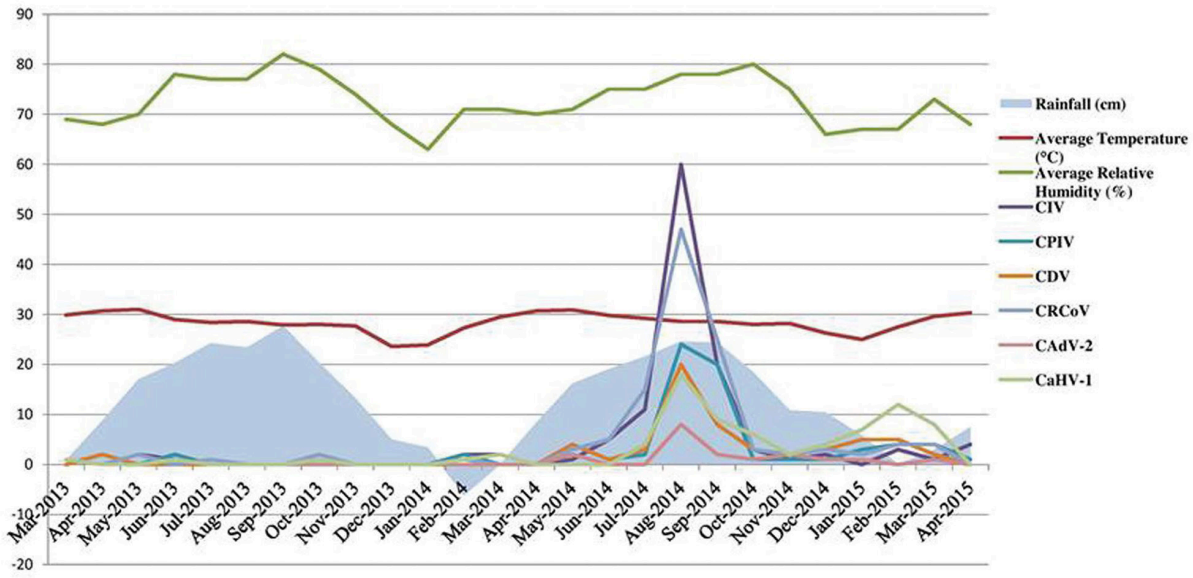

Fig. 4 Detection of CIRDC viruses in 209 respiratory ill dogs. Season was classified into the three periods of summer (March–June), rainy (July–October) and winter (November–February). Monthly percentage of CIRDC virus detections was compared with the monthly average temperature, relative humidity and amounts of rainfall during March 2013 to April 2015.

4. Metagenomic analysis of respiratory samples from dogs suffering respiratory disease.

Ten lung samples of respiratory suffering dogs, that were negative for common virus associated respiratory tract infection by using developed mPCRs, were performed the NGS, yielding more than 800,000 reads discovered from each samples. Subsequently, there were five samples which presented interesting pathogenic viral genomes. Interestingly, 2 viruses were discovered from those 5 samples, namely canine bocavirus-2 (CBoV-2) and canine circovirus (CanineCV).

4.1) Canine bocavirus-2

For CBoV-2, the virus was detected from three 10-day old Welsh Corgi pups from the same litter (16P177W-16P179W, Fig. 5A). All puppies presented depression with severe dyspnea, followed by hemoptysis. Oxygen therapy, antibiotic and intensive bronchodilators were treated. The clinical signs were getting worse and the puppy was died within 3 hours after treatment. Later, the other 4 puppies in the same litter were consecutively died with severe respiratory distress and hemoptysis within 2 days; however a completed core-vaccinated dam was asymptomatic. Postmortem examination revealed gross morphology showing bloody nasal discharge. Various degrees of red hepatization of cranial and caudal lung lobes were observed (Fig. 5B). One puppy showed adhesion between rib and left lung lobe. The kidneys were bulgy on cut surface with pale color; however, renal cortex swelling with patchy hemorrhage at cortico-medullary junction was noted in one puppy. Spleen and liver represented dark red color with mild degree of blunt edges. Few brownish mucous with milk curd were noted along stomach and small intestine. Microscopically, the lung of three puppies represented severe, acute, diffuse alveolar edema with multifocal, histiocytosis, hemorrhage, hyperemia and emphysema. High number of intra-alveolar bacteria was noted in two puppies; however, there was no inflammatory cell infiltration in the lesions (Fig. 5C). Moderate, acute, diffuse, hyperemia was presented in liver of all puppies. Numerous eosinophilic intranuclear inclusion bodies (INCB) were presented in enterocyte without any other lesions in two puppies, although neither INCB nor lesions were observed in another one puppy (Fig. 5D). Moderate sinus histiocytosis was often observed in lymph nodes. Spleen, kidneys, trachea and heart were non-remarkable lesion in all puppies.

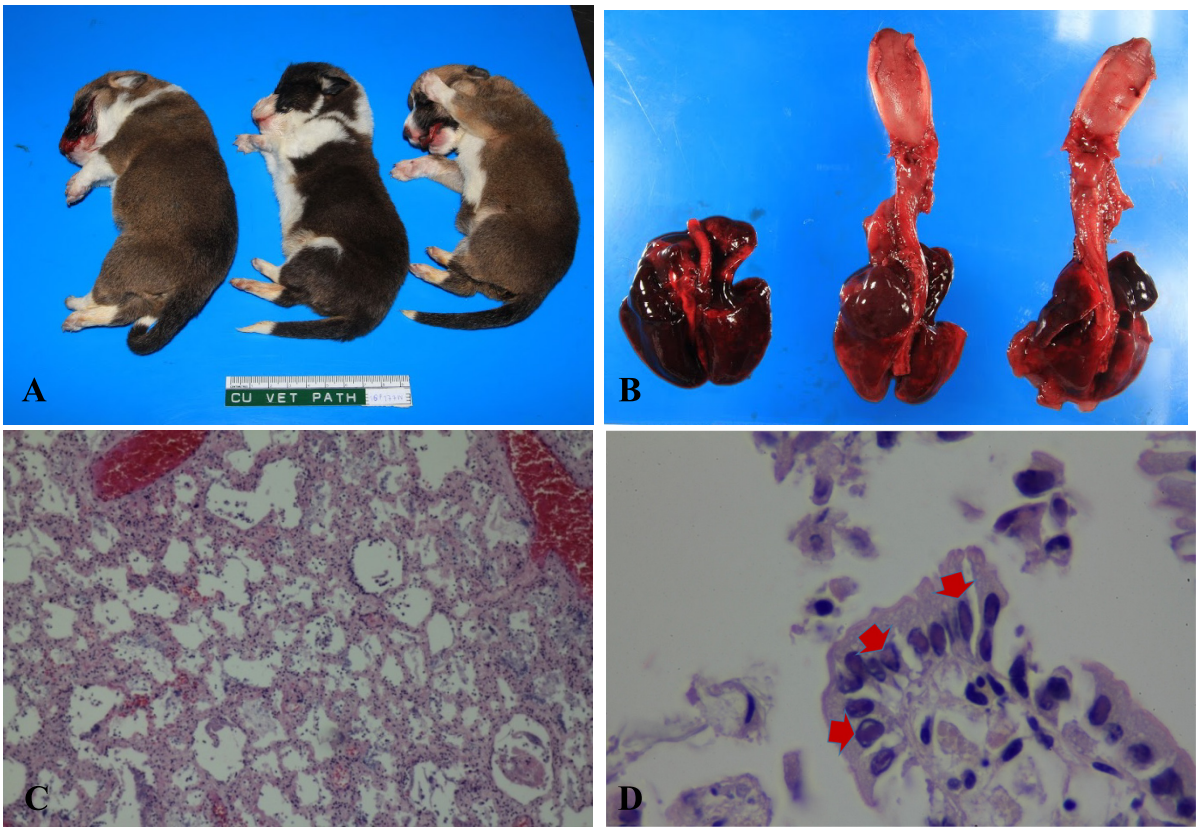

Fig. 5 Canine bocavirus type-2 infection in puppies. (A) Three 10-day old Welsh Corgi pups from the same litter (16P177W-16P179W), (B) Severe acute diffuse pneumonia with red hepatization, (C) Severe acute diffuse necrotizing and suppurative pneumonia, (D) Eosinophilic intranuclear inclusion bodies in enterocytes (red arrows)

For the NGS result, total 832,912, 857,945 and 1,037,842 qualified trimmed reads were obtained from lung samples of individual puppy no. 1 to 3, respectively. Averaged 50 reads of each puppy were identity to CBoV-2 in various strains. All identical CBoV-2 sequences derived from each puppy were aligned and pooled, then constructed for its genome. The NGS-derived CBoV-2 sequence gaps were filled by using conventional PCRs, obtained from developed primers (Table 9), and Sanger sequencing. The constructed CBoV-2 sequence was tentatively named as CBoV TH-2016. The 5,127 bp of near complete genome sequence of CBoV TH-2016 was established. It contained three main open reading frames (ORFs). The ORF1 coded the overlapping nonstructural protein (NS); NS1 (nt 226-2607) and NS2 (nt 226-2137, nt 2212-2607), encoding 793 and 637 amino acids. The ORF2 coded the overlapping viral capsid protein (VP) 1 (nt 2943-5060) and VP2 (nt 3357-5060), coding 705 and 567 amino acids. The ORF3 coded a nucleoprotein (NP) (nt 2372-2959), coding 195

amino acids. Furthermore, one additional ORF, ORF4 (nt 2173-2607), was detected in this sequence, downstream after the NS1 (Fig. 6). Phylogenetic tree of complete genome of CBoV in various strains exhibited the CBoV TH-2016 was 93.3% nucleotides and 87.8% deduced amino acids closely related to the CBoV strains of South Korea (Accession number: KP281718) (Fig. 7). Moreover, deduced amino acids based phylogenetic tree of VP1/2 and NS1 revealed that the CBoV TH-2016 was closely related to South Korea and Hong Kong strains, respectively (Fig. 8, 9).

Table 9 CBoV-2 specific primers used for complete genome sequencing.

Genome position[#] (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	

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

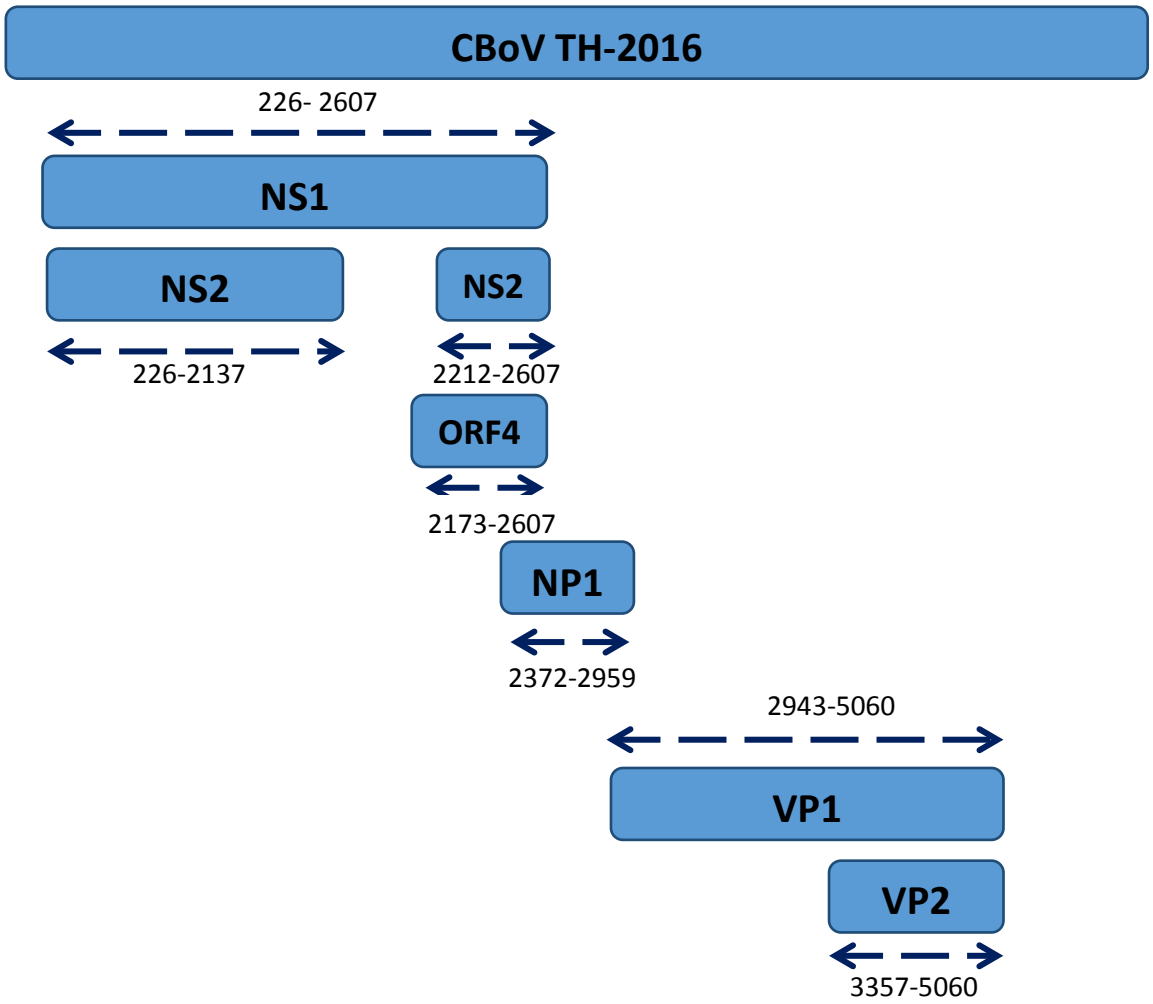

Fig. 6 Schematic of nearly CBoV TH-2016 genome and phylogenetic analysis. (A) Genome organization of novel canine bocavirus strain CBoV TH-2016 coding NS1, NS2, NP1, ORF4, and VP1/2.

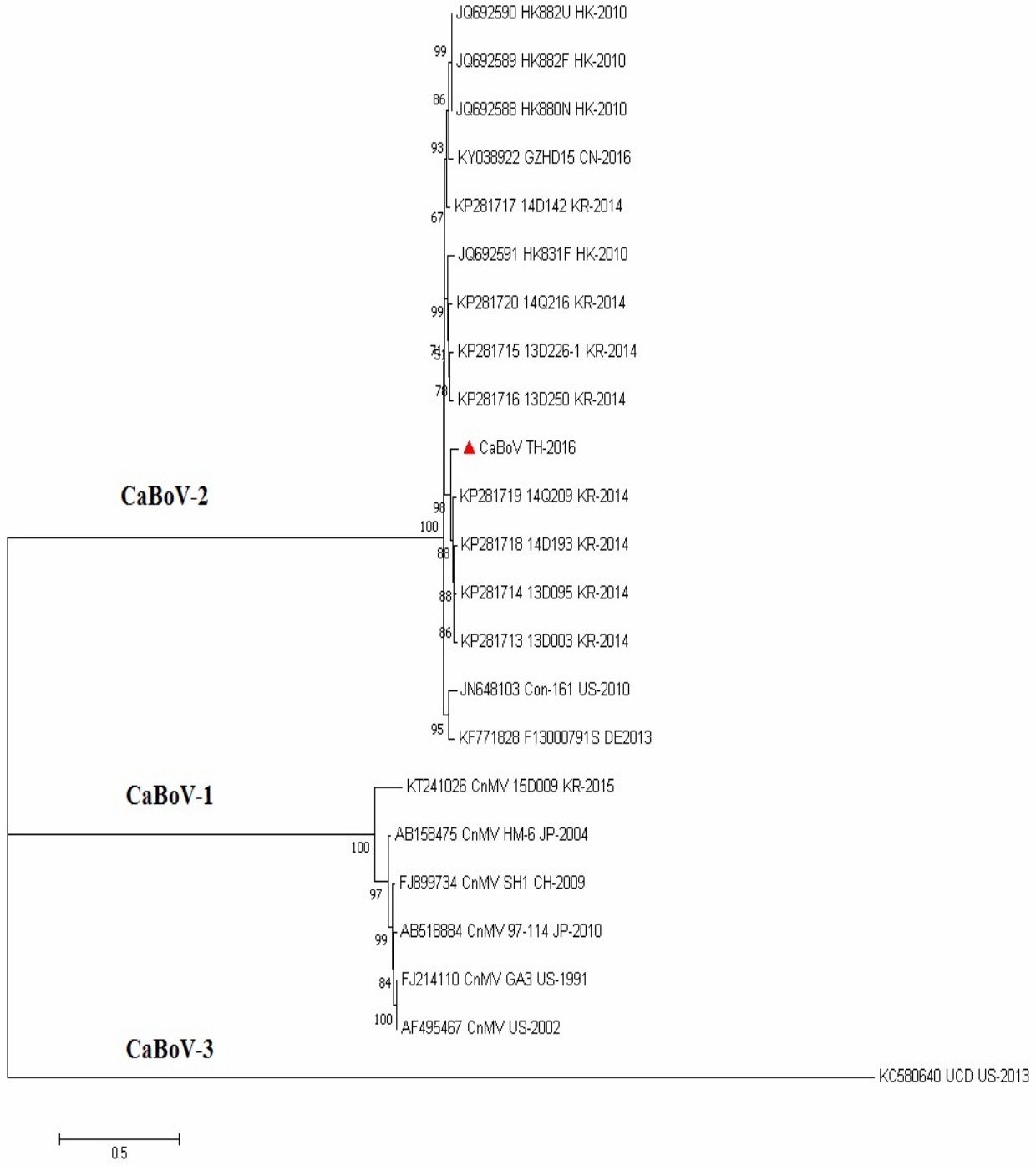

Fig. 7 Phylogenetic tree of nearly complete CBoV TH-2016 genome compared with various *Bocaparvovirus* and constructed by Maximum-likelihood algorithm with 1000 bootstrap replicates. CnMV: canine minute virus.

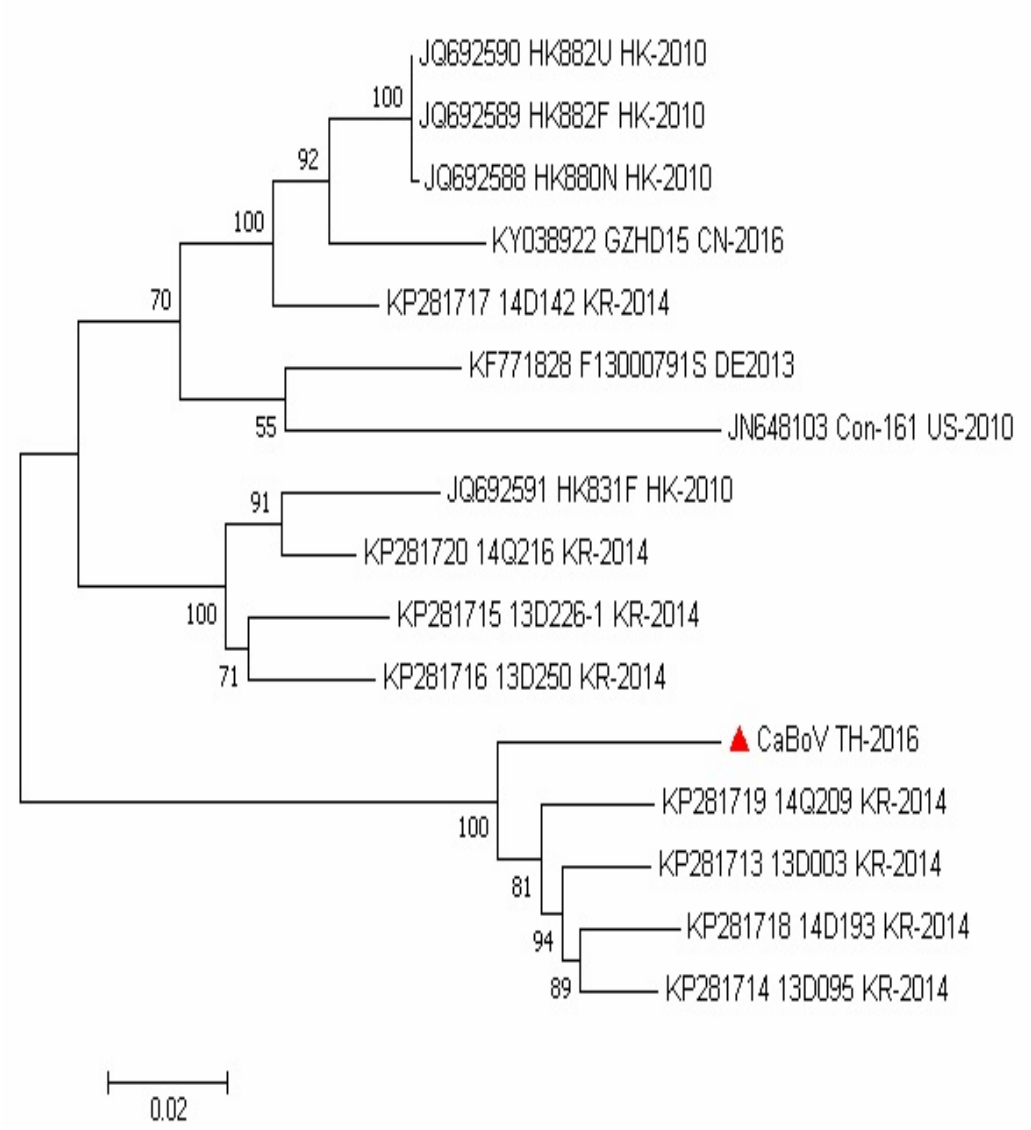

Fig. 8 Phylogenetic tree of CBoV TH-2016 VP1/2 gene compared with various *Bocaparvovirus* and constructed by Maximum-likelihood algorithm with 1000 bootstrap replicates.

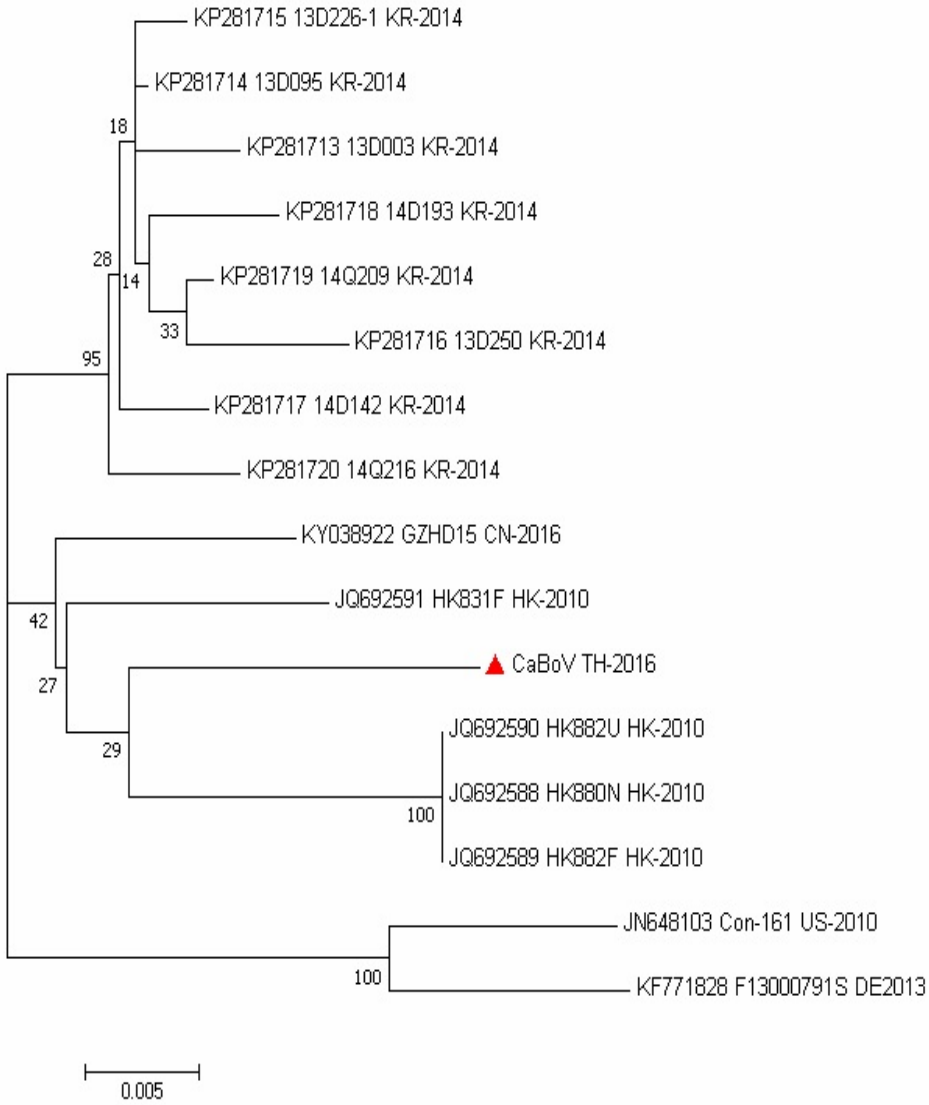

Fig. 9 Phylogenetic tree of CBoV TH-2016 NS1 gene compared with various *Bocaparvovirus* and constructed by Maximum-likelihood algorithm with 1000 bootstrap replicates.

Additionally, a unique deletion of 18 nucleotides was presented in VP1/2 gene (Fig. 10). The NS1/2, NP, and VP1/2 genomes of CBoV TH-2016 were calculated for pair-wise distances with 14 CBoV strains (Accession numbers: KP281717, KP281714, KP281715, KP281713, KP281718, KP281719, KP281720, KP281716, JQ692591, JQ692590, JQ692588, JQ692589, JN648103 and KF771828). The results showed that up to 20.8%, 3.3%, 12.3% (average: 9%, 1.7%, 8.1%) nucleotides and 20.1%, 1.6%, 9.2% (average: 9.1%, 1.2%, 5.7%) amino acids diversities, respectively. The ORF4 was also calculated the pair-wise distances with 9 CBoV-2 variants (Accession number: JQ692588, JQ692589, JQ692590, JQ692591, KP281715, KP281716, KP281718, KP281719, KP281720), resulted up to 2.3% (2.5% average variations) nucleotides and 2.1% (2.6 average variations) amino acids (Fig. 11-14).

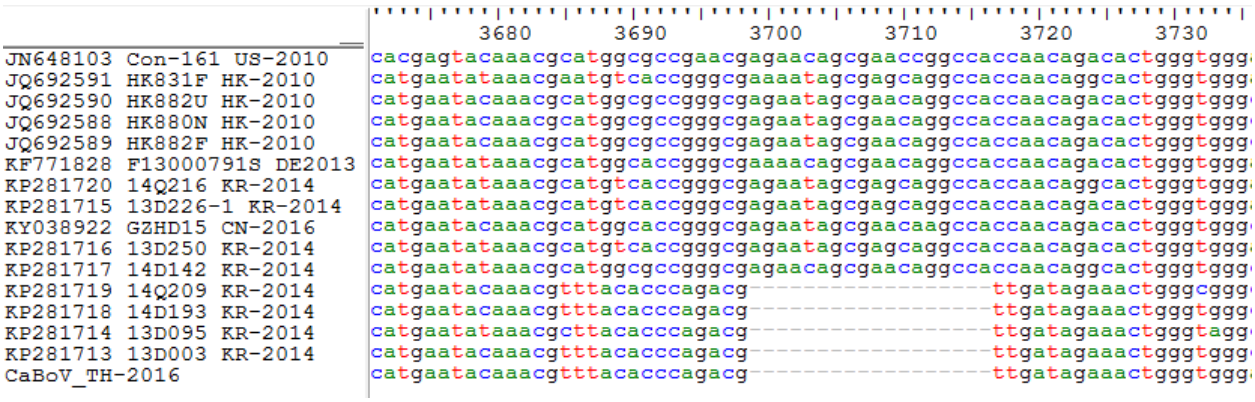

Fig. 10 A deletion of 18 nucleotides that found in 14Q209, 14D193, 13D095 and 13D003 strains of CBoV-2 between nucleotides 3,699-3,716 and located at VP1/2 was also presented in CBoV TH-2016.

Seq->	HK831F	HK882U	HK880N	HK882F	14Q216	13D226-1	13D250	Con-161	F13000791S	14D142	14Q209	14D193	13D095	13D003	GZHD15	TH-2016
HK831F	ID	0,943	0,954	0,939	0,939	0,933	0,932	0,872	0,933	0,923	0,912	0,907	0,906	0,883	0,933	0,917
HK882U	0,943	ID	0,987	0,972	0,933	0,933	0,933	0,884	0,934	0,949	0,921	0,916	0,919	0,894	0,938	0,928
HK-2010	0,954	0,987	ID	0,983	0,923	0,923	0,923	0,875	0,944	0,939	0,911	0,906	0,908	0,884	0,949	0,92
HK882F	0,939	0,972	0,983	ID	0,909	0,909	0,909	0,891	0,939	0,925	0,897	0,892	0,894	0,88	0,951	0,918
14Q216	0,939	0,933	0,923	0,909	ID	0,977	0,976	0,899	0,907	0,964	0,954	0,949	0,948	0,913	0,902	0,907
13D226-1	0,933	0,933	0,923	0,909	0,977	ID	0,977	0,895	0,904	0,966	0,959	0,953	0,952	0,917	0,901	0,91
13D250	0,932	0,933	0,923	0,909	0,976	0,977	ID	0,899	0,905	0,964	0,954	0,949	0,947	0,912	0,901	0,909
Con-161	0,872	0,884	0,875	0,891	0,899	0,895	0,899	ID	0,897	0,899	0,887	0,886	0,885	0,915	0,872	0,874
F13000791S	0,933	0,934	0,944	0,939	0,907	0,904	0,905	0,897	ID	0,915	0,895	0,89	0,894	0,881	0,93	0,912
14D142	0,923	0,949	0,939	0,925	0,964	0,966	0,964	0,899	0,915	ID	0,954	0,949	0,949	0,916	0,915	0,912
14Q209	0,912	0,921	0,911	0,897	0,954	0,959	0,954	0,887	0,895	0,954	ID	0,978	0,979	0,942	0,889	0,932
14D193	0,907	0,916	0,906	0,892	0,949	0,953	0,949	0,886	0,89	0,949	0,978	ID	0,979	0,941	0,886	0,933
13D095	0,906	0,919	0,908	0,894	0,948	0,952	0,947	0,885	0,894	0,949	0,979	0,979	ID	0,949	0,886	0,932
13D003	0,883	0,894	0,884	0,88	0,913	0,917	0,912	0,915	0,881	0,916	0,942	0,941	0,949	ID	0,87	0,916
GZHD15	0,933	0,938	0,949	0,951	0,902	0,901	0,901	0,872	0,93	0,915	0,889	0,886	0,886	0,87	ID	0,91
CaBoV_TH-2016	0,917	0,928	0,92	0,918	0,907	0,91	0,909	0,874	0,912	0,912	0,932	0,933	0,932	0,916	0,91	ID

Fig. 11 Pairwise identity matrix measured genetically similarity nucleotides of nearly complete CBoV TH-2016 genome compared with other CBoV strains.

Seq->	HK831F	HK882U	HK880N	HK882F	14Q216	13D226-1	13D250	Con-161	F13000791S	14D142	14Q209	14D193	13D095	13D003	GZHD15	TH-2016
HK831F	ID	0,975	0,975	0,975	0,948	0,946	0,975	0,964	0,935	0,947	0,953	0,797	0,797	0,937	0,798	0,794
HK882U	0,975	ID	1	1	0,949	0,951	0,98	0,968	0,939	0,952	0,957	0,799	0,801	0,938	0,798	0,797
HK880N	0,975	1	ID	1	0,949	0,951	0,98	0,968	0,939	0,952	0,957	0,799	0,801	0,938	0,798	0,797
HK882F	0,975	1	1	ID	0,949	0,951	0,98	0,968	0,939	0,952	0,957	0,799	0,801	0,938	0,798	0,797
14Q216	0,948	0,949	0,949	0,949	ID	0,99	0,96	0,945	0,964	0,99	0,984	0,83	0,826	0,97	0,823	0,819
13D226-1	0,946	0,951	0,951	0,951	0,99	ID	0,96	0,943	0,962	0,991	0,986	0,832	0,829	0,972	0,823	0,82
13D250	0,975	0,98	0,98	0,98	0,96	0,96	ID	0,97	0,94	0,959	0,969	0,808	0,809	0,949	0,801	0,795
Con-161	0,964	0,968	0,968	0,968	0,945	0,943	0,97	ID	0,952	0,946	0,95	0,793	0,795	0,931	0,793	0,792
F13000791S	0,935	0,939	0,939	0,939	0,964	0,962	0,94	0,952	ID	0,963	0,957	0,809	0,814	0,956	0,81	0,807
14D142	0,947	0,952	0,952	0,952	0,99	0,991	0,959	0,946	0,963	ID	0,986	0,83	0,829	0,971	0,823	0,819
14Q209	0,953	0,957	0,957	0,957	0,984	0,986	0,969	0,95	0,957	0,986	ID	0,825	0,824	0,968	0,817	0,814
14D193	0,797	0,799	0,799	0,799	0,83	0,832	0,808	0,793	0,809	0,83	0,825	ID	0,982	0,83	0,978	0,977
13D095	0,797	0,801	0,801	0,801	0,826	0,829	0,809	0,795	0,814	0,829	0,824	0,982	ID	0,845	0,978	0,972
13D003	0,937	0,938	0,938	0,938	0,97	0,972	0,949	0,931	0,956	0,971	0,968	0,83	0,845	ID	0,825	0,82
GZHD15	0,798	0,798	0,798	0,798	0,823	0,823	0,801	0,793	0,81	0,823	0,817	0,978	0,978	0,825	ID	0,978
TH-2016	0,794	0,797	0,797	0,797	0,819	0,82	0,795	0,792	0,807	0,819	0,814	0,977	0,972	0,82	0,978	ID

Fig. 12 Pairwise identity matrix measured genetically similarity nucleotides of CBoV TH-2016 NS1 gene compared with other CBoV strains.

Seq->	HK831F	HK882U	HK880N	HK882F	14Q216	13D226-1	13D250	Con-161	F130007915	14D142	14Q209	14D193	13D095	13D003	GZHD15	TH-2016
HK831F	ID	0,926	0,926	0,926	0,964	0,953	0,948	0,911	0,918	0,927	0,902	0,888	0,892	0,894	0,926	0,889
HK882U	0,926	ID	0,999	1	0,928	0,926	0,923	0,913	0,94	0,965	0,896	0,887	0,892	0,895	0,964	0,891
HK880N	0,926	0,999	ID	0,999	0,928	0,925	0,923	0,912	0,94	0,964	0,895	0,886	0,892	0,894	0,964	0,89
HK882F	0,926	1	0,999	ID	0,928	0,926	0,923	0,913	0,94	0,965	0,896	0,887	0,892	0,895	0,964	0,891
14Q216	0,964	0,928	0,928	0,928	ID	0,959	0,963	0,917	0,922	0,931	0,905	0,897	0,897	0,899	0,926	0,886
13D226-1	0,953	0,926	0,925	0,926	0,959	ID	0,962	0,911	0,918	0,934	0,913	0,904	0,902	0,904	0,923	0,891
13D250	0,948	0,923	0,923	0,923	0,963	0,962	ID	0,92	0,92	0,934	0,904	0,896	0,896	0,898	0,922	0,887
Con-161	0,911	0,913	0,912	0,913	0,917	0,911	0,92	ID	0,927	0,916	0,89	0,885	0,879	0,886	0,911	0,877
F130007915	0,918	0,94	0,94	0,94	0,922	0,918	0,92	0,927	ID	0,939	0,895	0,883	0,887	0,893	0,935	0,89
14D142	0,927	0,965	0,964	0,965	0,931	0,934	0,934	0,916	0,939	ID	0,905	0,895	0,898	0,903	0,955	0,897
14Q209	0,902	0,896	0,895	0,896	0,905	0,913	0,904	0,89	0,895	0,905	ID	0,966	0,969	0,968	0,894	0,947
14D193	0,888	0,887	0,886	0,887	0,897	0,904	0,896	0,885	0,883	0,895	0,966	ID	0,973	0,969	0,885	0,948
13D095	0,892	0,892	0,892	0,892	0,897	0,902	0,896	0,879	0,887	0,898	0,969	0,973	ID	0,974	0,887	0,951
13D003	0,894	0,895	0,894	0,895	0,899	0,904	0,898	0,886	0,893	0,903	0,968	0,969	0,974	ID	0,892	0,952
GZHD15	0,926	0,964	0,964	0,964	0,926	0,923	0,922	0,911	0,935	0,955	0,894	0,885	0,887	0,892	ID	0,891
TH-2016	0,889	0,891	0,89	0,891	0,886	0,891	0,887	0,877	0,89	0,897	0,947	0,948	0,951	0,952	0,891	ID

Fig. 13 Pairwise identity matrix measured genetically similarity nucleotides of CBoV TH-2016 VP1/2 gene compared with other CBoV strains.

Seq->	HK831F	HK882U	HK880N	HK882F	14Q216	13D226-1	KR-2014	Con-161	F130007915	14D142	14Q209	14D193	13D095	13D003	GZHD15	TH-2016
HK831F	ID	0,986	0,986	0,986	0,982	0,982	0,982	0,969	0,965	0,986	0,982	0,988	0,982	0,984	0,994	0,984
HK882U	0,986	ID	1	1	0,982	0,989	0,986	0,969	0,962	0,986	0,989	0,988	0,989	0,988	0,988	0,991
HK880N	0,986	1	ID	1	0,982	0,989	0,986	0,969	0,962	0,986	0,989	0,988	0,989	0,988	0,988	0,991
HK882F	0,986	1	1	ID	0,982	0,989	0,986	0,969	0,962	0,986	0,989	0,988	0,989	0,988	0,988	0,991
14Q216	0,982	0,982	0,982	0,982	ID	0,986	0,982	0,965	0,959	0,982	0,982	0,988	0,986	0,984	0,984	0,984
13D226-1	0,982	0,989	0,989	0,989	0,986	ID	0,993	0,969	0,962	0,986	0,996	0,988	0,989	0,988	0,988	0,988
13D250	0,982	0,986	0,986	0,986	0,982	0,993	ID	0,969	0,962	0,986	0,993	0,991	0,986	0,988	0,988	0,994
Con-161	0,969	0,969	0,969	0,969	0,965	0,969	0,969	ID	0,993	0,972	0,965	0,974	0,969	0,971	0,974	0,974
F130007915	0,965	0,962	0,962	0,962	0,959	0,962	0,962	0,993	ID	0,969	0,959	0,967	0,962	0,964	0,971	0,967
14D142	0,986	0,986	0,986	0,986	0,982	0,986	0,986	0,972	0,969	ID	0,986	0,994	0,989	0,991	0,991	0,988
14Q209	0,982	0,989	0,989	0,989	0,982	0,996	0,993	0,965	0,959	0,986	ID	0,988	0,989	0,984	0,988	0,988
14D193	0,988	0,988	0,988	0,988	0,988	0,988	0,991	0,974	0,967	0,994	0,988	ID	0,991	0,993	0,993	0,993
13D095	0,982	0,989	0,989	0,989	0,986	0,989	0,986	0,969	0,962	0,989	0,989	0,991	ID	0,988	0,988	0,988
13D003	0,984	0,988	0,988	0,988	0,984	0,988	0,988	0,971	0,964	0,991	0,984	0,993	0,988	ID	0,986	0,989
GZHD15	0,994	0,988	0,988	0,988	0,984	0,988	0,988	0,974	0,971	0,991	0,988	0,993	0,988	0,986	ID	0,989
TH-2016	0,984	0,991	0,991	0,991	0,984	0,988	0,994	0,974	0,967	0,988	0,988	0,993	0,988	0,989	0,989	ID

Fig. 14 Pairwise identity matrix measured genetically similarity nucleotides of CBoV TH-2016 NP gene compared with other CBoV strains.

4.1) Canine circovirus

For canine circovirus (CanineCV), there were 2 dogs detected by NGS. Briefly, Dog 1 (14P105D): A 4-month-old vaccinated male Pomeranian was kenneled at private clinic, then showed suddenly severe depression, followed by soft stool diarrhea and hemoptysis. Although the dog was treated by empirical antibiotics and fluid therapy, his clinical signs were not improved. The dog died after progressive hemoptysis could not be controlled.

Dog 2 (14P112N): A 1-month-old female Labrador retriever, delivered from vaccinated breeding colony, showed bloody vomiting with mucopurulent nasal discharge. The dog was treated by nebulization and fluid therapy. Respiratory clinical signs were not improved then the dog was sudden dead with acute apnea. Furthermore, other puppies in the same litter were following dead with the same clinical signs.

Gross and histological examination in these two dogs revealed various degrees of pneumonia by showing severe reddish and gray in color of caudal and cranial lung lobes, respectively (Fig. 15 A, B). Moderate multifocal acute hemorrhage with alveolar emphysema and moderate multifocal suppurative bronchopneumonia along with multifocal alveolar histiocytosis were noted in dog 1. Severe diffuse suppurative fibrinous bronchopneumonia was presented in dog 2 (Fig. 15 C, D). Mild lymphoid hyperplasia of tracheobronchial lymph node and tonsil were examined in both dogs.

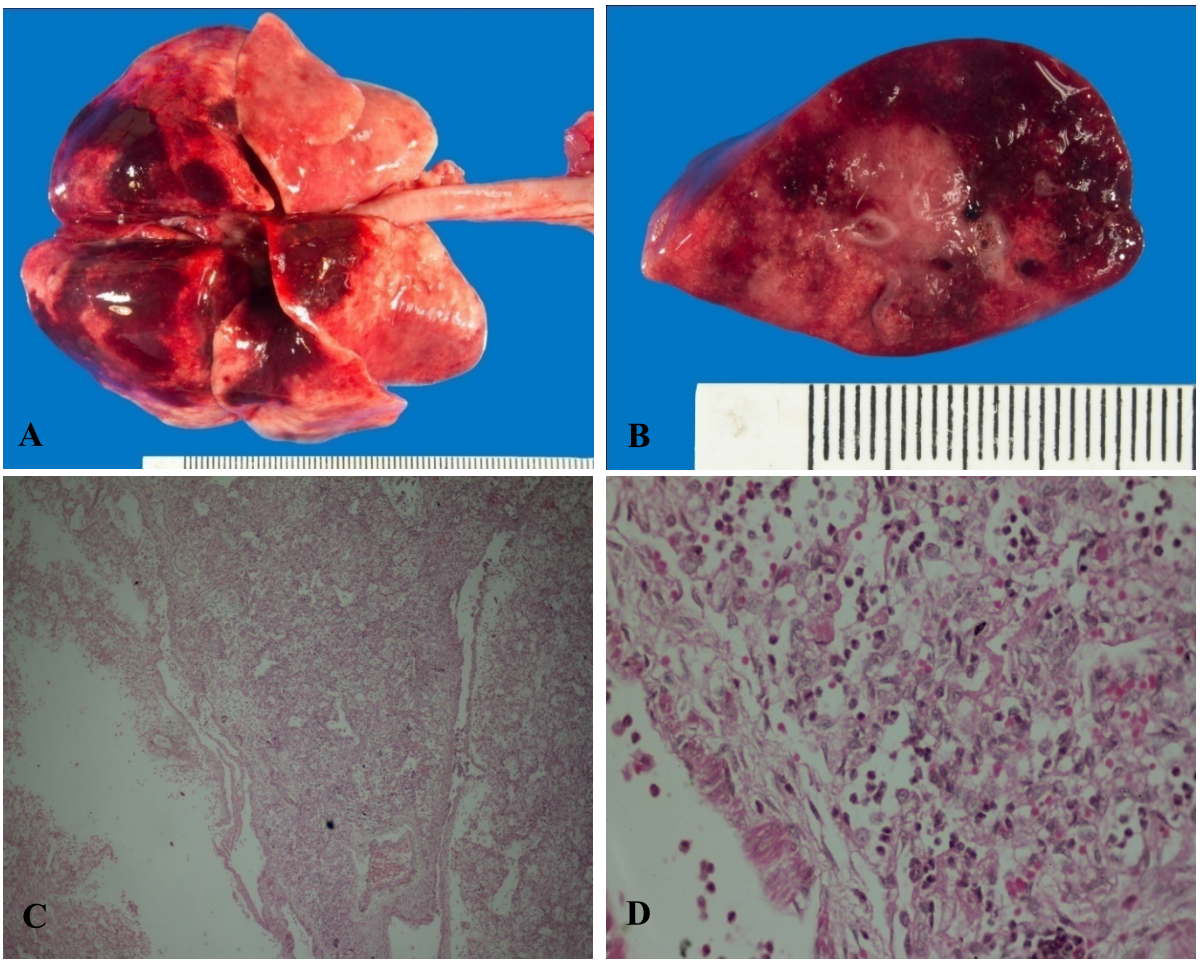

Fig. 15 Canine circovirus infection in puppies. (A, B) Gross lesion of lung from a 4-month-old puppy (14P105D), severe subacute locally extensive pneumonia, (C, D) Histopathological lesion of lung from a 1-month-old puppy (14P112N), severe diffuse suppurative bronchopneumonia

In term of NGS results, over 900,000 trimmed reads were obtained from individual lung samples and more than 380 and 10 reads were detected from dog 1 and dog 2, respectively. The highest reads had similarity to CanineCV without any other pathogenic viruses were detected. However, the discontinuations of CanineCV sequence outputs from the NGS were presented. Therefore, gaps between sequences were filled with sequence-based PCR reactions by using designed primer derived from the NGS results (Table 10).

Table 10 Sequences of canine circovirus specific primers used for complete genome analysis

Genome position (nucleotide)	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	

* Additional primers used for double amplifying Canine CV capsid gene region

The NGS-derived CanineCV from both dogs, namely CanineCV_14P105D-TH2016 and CanineCV_14P112N-TH2016, were confirmed by PCR. The coding sequence of the CanineCV 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 304 amino acids and the Cap gene, nt 1,116-1,928, encoded 271 amino acids (Fig. 16). Pairwise identical matrix revealed that the CanineCV_14P105D-TH2016 and 14P112N-TH2016 had genetic similarity to CanineCV UCD3-478 strain, accounted for 86.6 and 81.1%, respectively whereas 82.0% and 77.1% genetic identity to CanineCV AZ-5586-13 strain (Fig. 17). Phylogenetic analysis of CanineCV detecting in Thailand was constructed based on nearly complete sequences, suggesting the CanineCV_14P105D-TH2016 and 14P112N-TH2016 strains were grouped in the new clade of CanineCV and closely related to CanineCV UCD3-478 strain (Fig. 18). The individual Rep and Cap genes were used to construct the phylogenetic tree, revealing both CanineCV strains found in Thailand were clustered with CanineCV UCD3-478 for the Cap (Fig. 19), but not for the Rep which clustered away from CanineCV UCD3-478 strain as a new clade (Fig. 20).

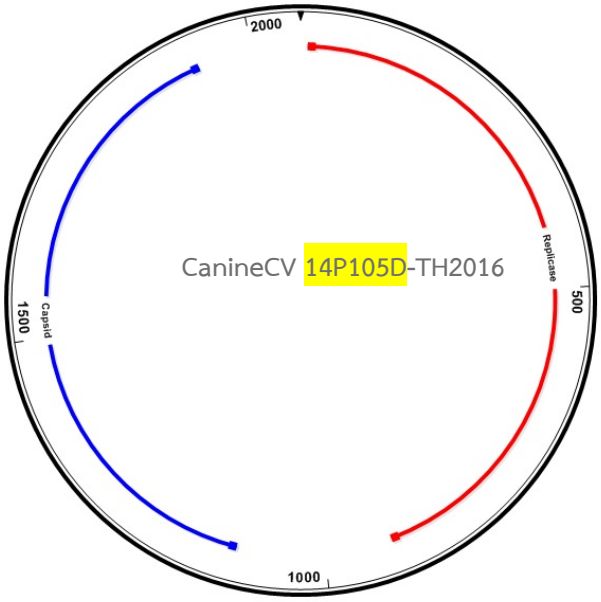

Fig. 16 Genome organization of canine circovirus strain 14P105D-TH2016.

Seq->	UCD1-16	UCD2-3:	Bari/411	AZ4133/	AZ4133/	TE4016-	AZ663/1	AZ663/2	AZ5212/	AZ5212/	AZ5586-	TE7482	FUBerlin	AZ2972-	AZ4438-	Ha13	CB629:	CB6293	PE8575/	PE8575/	isolate :	TE6685	TE6685	JZ98/20	UCD3-4	14P105E	14P112N
UCD1-1638	ID	0,952	0,952	0,951	0,951	0,952	0,956	0,956	0,951	0,951	0,95	0,948	0,946	0,955	0,943	0,948	0,966	0,966	0,964	0,964	0,965	0,935	0,935	0,895	0,868	0,843	0,784
UCD2-32162	0,952	ID	0,98	0,978	0,978	0,977	0,98	0,98	0,974	0,973	0,977	0,976	0,967	0,972	0,972	0,972	0,964	0,964	0,962	0,962	0,961	0,958	0,958	0,899	0,854	0,828	0,78
Bari/411-13	0,952	0,98	ID	0,987	0,987	0,982	0,983	0,983	0,981	0,98	0,981	0,98	0,968	0,971	0,975	0,971	0,96	0,96	0,96	0,96	0,961	0,956	0,956	0,899	0,848	0,824	0,775
AZ4133/1-13	0,951	0,978	0,987	ID	1	0,98	0,981	0,981	0,977	0,976	0,979	0,979	0,968	0,97	0,975	0,972	0,96	0,96	0,958	0,958	0,962	0,956	0,956	0,898	0,848	0,824	0,775
AZ4133/2-13	0,951	0,978	0,987	1	ID	0,98	0,981	0,981	0,977	0,976	0,979	0,979	0,968	0,97	0,975	0,972	0,96	0,96	0,958	0,958	0,962	0,956	0,956	0,898	0,848	0,824	0,775
TE4016-13	0,952	0,977	0,982	0,98	0,98	ID	0,984	0,984	0,984	0,984	0,98	0,978	0,966	0,967	0,97	0,968	0,96	0,96	0,959	0,959	0,961	0,954	0,954	0,896	0,849	0,825	0,776
AZ663/1-13	0,956	0,98	0,983	0,981	0,981	0,984	ID	1	0,982	0,981	0,984	0,981	0,97	0,972	0,975	0,972	0,965	0,965	0,963	0,963	0,963	0,957	0,957	0,898	0,852	0,827	0,778
AZ663/2-13	0,956	0,98	0,983	0,981	0,981	0,984	1	ID	0,982	0,981	0,984	0,981	0,97	0,972	0,975	0,972	0,965	0,965	0,963	0,963	0,963	0,957	0,957	0,898	0,852	0,827	0,778
AZ5212/1-14	0,951	0,974	0,981	0,977	0,977	0,984	0,982	0,982	ID	0,999	0,982	0,978	0,968	0,968	0,969	0,966	0,96	0,96	0,959	0,959	0,961	0,952	0,952	0,898	0,851	0,823	0,774
AZ5212/2-14	0,951	0,973	0,98	0,976	0,976	0,984	0,981	0,981	0,999	ID	0,982	0,977	0,968	0,968	0,969	0,965	0,96	0,96	0,958	0,958	0,96	0,952	0,952	0,898	0,85	0,822	0,774
AZ5586-13	0,95	0,977	0,981	0,979	0,979	0,98	0,984	0,984	0,982	0,982	ID	0,979	0,966	0,969	0,972	0,968	0,96	0,96	0,96	0,96	0,962	0,955	0,955	0,896	0,846	0,82	0,771
TE7482-13	0,948	0,976	0,98	0,979	0,979	0,978	0,981	0,981	0,978	0,977	0,979	ID	0,967	0,969	0,971	0,968	0,958	0,958	0,957	0,957	0,958	0,953	0,953	0,898	0,847	0,823	0,774
FUBerlin-JRS	0,946	0,967	0,968	0,968	0,968	0,966	0,97	0,97	0,968	0,968	0,966	0,967	ID	0,982	0,983	0,98	0,959	0,959	0,955	0,955	0,955	0,955	0,955	0,894	0,846	0,825	0,777
AZ2972-13	0,955	0,972	0,971	0,97	0,97	0,967	0,972	0,972	0,968	0,968	0,963	0,963	0,982	ID	0,985	0,98	0,965	0,965	0,963	0,963	0,963	0,96	0,96	0,9	0,852	0,83	0,781
AZ4438-13	0,949	0,972	0,975	0,975	0,975	0,97	0,975	0,975	0,969	0,969	0,972	0,971	0,983	0,985	ID	0,984	0,959	0,959	0,957	0,957	0,96	0,964	0,964	0,898	0,853	0,826	0,777
Ha13	0,948	0,972	0,971	0,972	0,972	0,968	0,972	0,972	0,966	0,965	0,968	0,968	0,98	0,98	0,984	ID	0,957	0,957	0,955	0,955	0,956	0,962	0,962	0,895	0,85	0,825	0,777
CB6293/1-14	0,966	0,964	0,96	0,96	0,96	0,96	0,965	0,965	0,96	0,96	0,96	0,958	0,959	0,965	0,959	0,957	ID	1	0,991	0,991	0,971	0,944	0,944	0,896	0,85	0,825	0,777
CB6293/2-14	0,966	0,964	0,96	0,96	0,96	0,96	0,965	0,965	0,96	0,96	0,96	0,958	0,959	0,965	0,959	0,957	1	ID	0,991	0,991	0,971	0,944	0,944	0,896	0,85	0,825	0,777
PE8575/1-13	0,964	0,962	0,96	0,958	0,958	0,959	0,963	0,963	0,959	0,958	0,96	0,957	0,955	0,963	0,957	0,955	0,991	0,991	ID	1	0,969	0,942	0,942	0,897	0,848	0,825	0,776
PE8575/2-13	0,964	0,962	0,96	0,958	0,958	0,959	0,963	0,963	0,959	0,958	0,96	0,957	0,955	0,963	0,957	0,955	0,991	0,991	1	ID	0,969	0,942	0,942	0,897	0,848	0,825	0,776
isolate 214	0,965	0,961	0,961	0,962	0,962	0,961	0,963	0,963	0,961	0,96	0,962	0,958	0,955	0,963	0,96	0,956	0,971	0,971	0,969	0,969	ID	0,944	0,944	0,899	0,854	0,827	0,779
TE6685/1-13	0,935	0,958	0,956	0,956	0,956	0,954	0,957	0,957	0,952	0,952	0,955	0,953	0,955	0,96	0,964	0,962	0,944	0,944	0,942	0,942	0,944	ID	1	0,898	0,862	0,835	0,783
TE6685/2-13	0,935	0,958	0,956	0,956	0,956	0,954	0,957	0,957	0,952	0,952	0,955	0,953	0,955	0,96	0,964	0,962	0,944	0,944	0,942	0,942	0,944	1	ID	0,898	0,862	0,835	0,783
JZ98/2014	0,895	0,899	0,899	0,898	0,898	0,896	0,898	0,898	0,898	0,898	0,896	0,898	0,894	0,9	0,898	0,895	0,896	0,896	0,897	0,897	0,899	0,898	0,898	ID	0,845	0,825	0,778
UCD3-478	0,868	0,854	0,848	0,848	0,848	0,849	0,852	0,852	0,851	0,85	0,846	0,847	0,846	0,852	0,853	0,85	0,85	0,85	0,848	0,848	0,854	0,862	0,862	0,845	ID	0,866	0,811
14P105D	0,843	0,828	0,824	0,824	0,824	0,825	0,827	0,827	0,823	0,822	0,82	0,823	0,825	0,83	0,826	0,825	0,825	0,825	0,825	0,825	0,827	0,835	0,835	0,825	0,866	ID	0,872
14P112N	0,784	0,78	0,775	0,775	0,775	0,776	0,778	0,778	0,774	0,774	0,771	0,774	0,777	0,781	0,777	0,777	0,777	0,777	0,776	0,776	0,779	0,783	0,783	0,778	0,811	0,872	ID

Fig. 17. Pairwise identity matrix measured genetically similarity nucleotides of canine circovirus strain 14P105D and 14P112N-TH2016 with other canine circovirus strains.

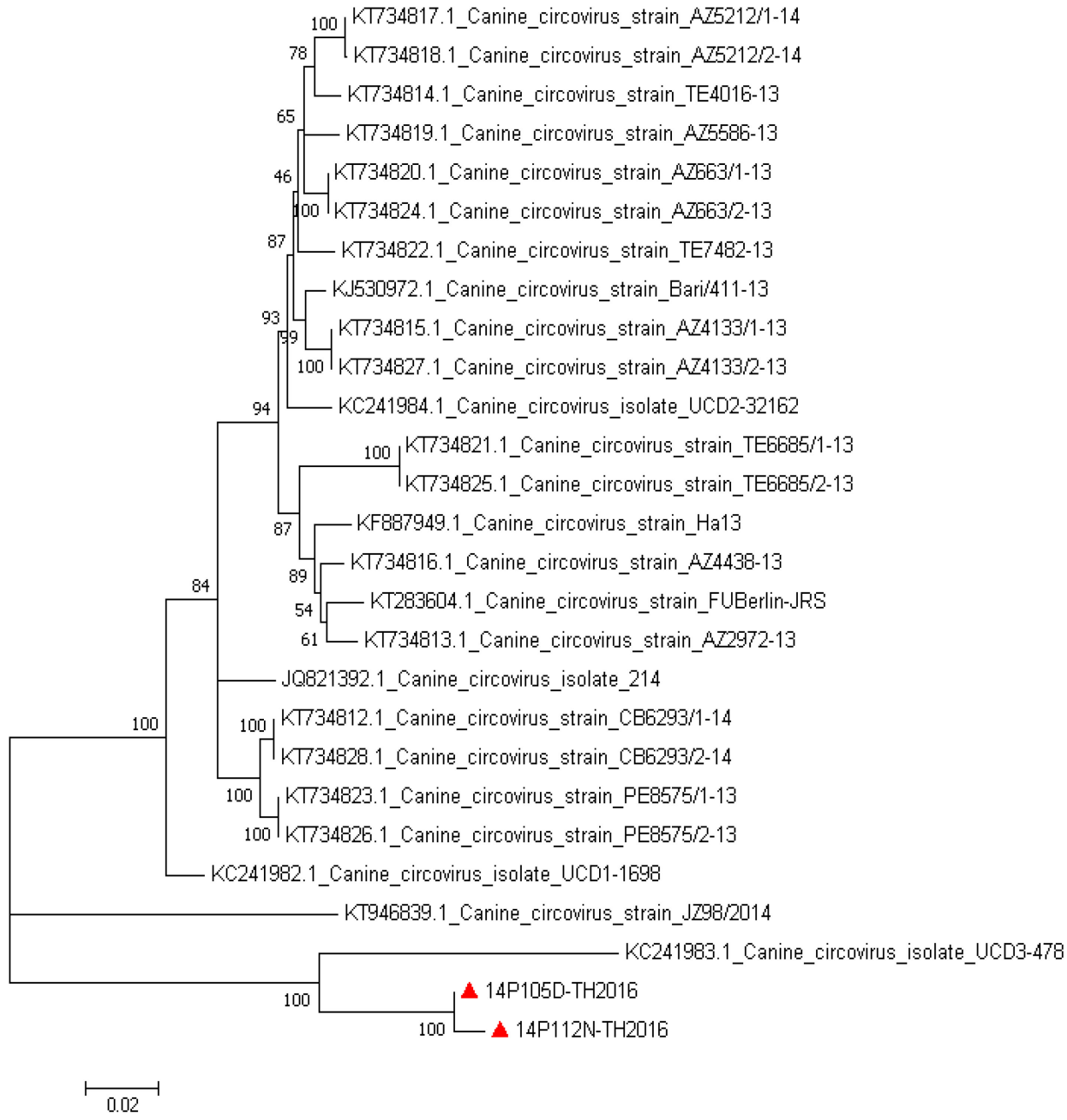

Fig. 18. Phylogenetic tree of canine circovirus complete genome. Canine circovirus strain 14P105D and 14P112N-TH2016 were labeled with ▲.

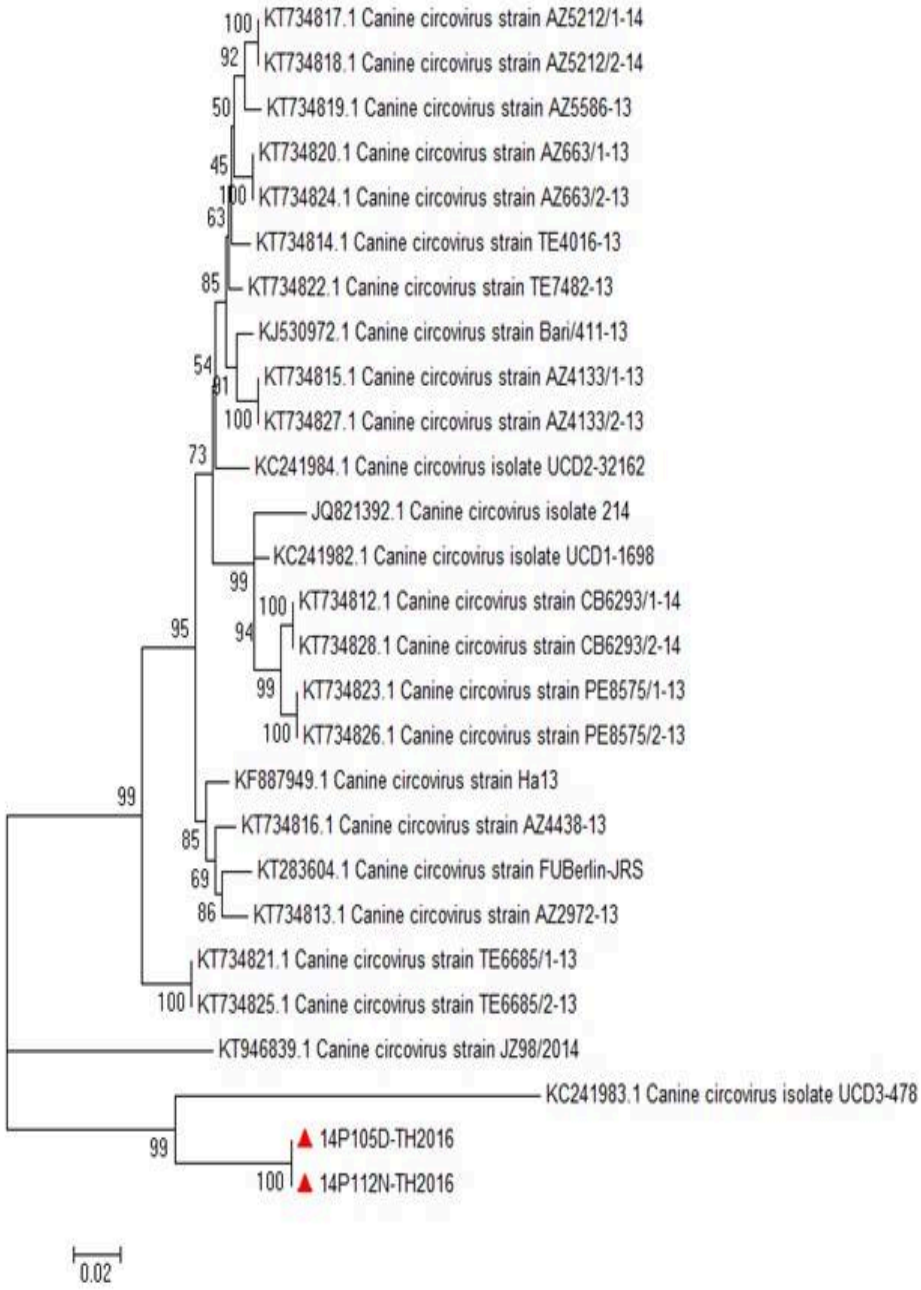

Fig. 19. Phylogenetic tree of canine circovirus constructed by Cap gene. Canine circovirus strain 14P105D and 14P112N-TH2016 were labeled with ▲.

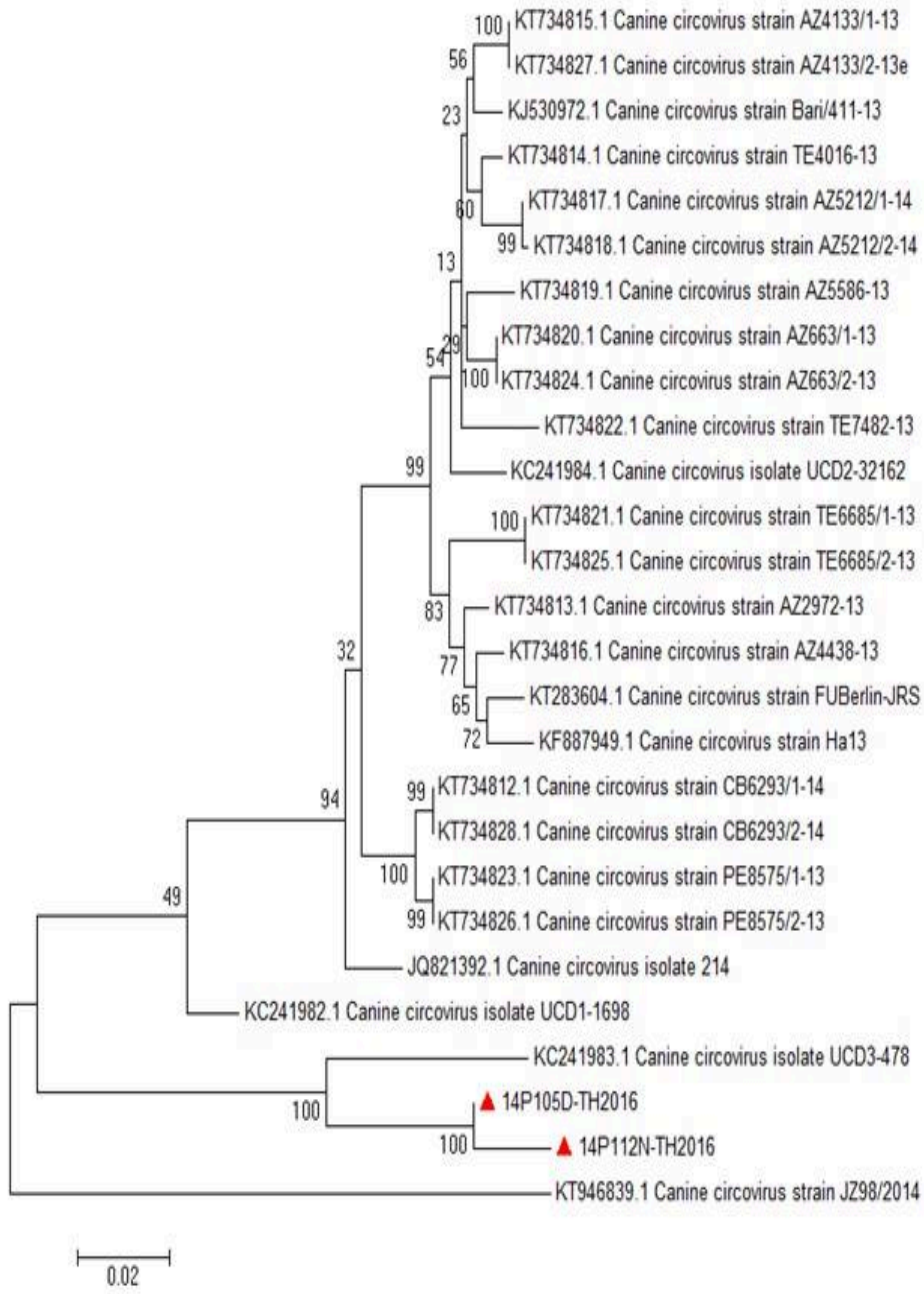

Fig. 20. Phylogenetic tree of canine circovirus constructed by Rep gene. Canine circovirus strain 14P105D and 14P112N-TH2016 were labeled with ▲.

V. Discussion

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 viruses detection 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 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 (Peceraro 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 (Mochizuki et al., 2008; Monteiro et al., 2016; Piewbang et al., 2016; Schulz et al., 2014; Viitanen et al., 2015). 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 (An et al., 2010; Buonavoglia and Martella, 2007; Chilvers et

al., 2001). Moreover, co-infection of CIV and CRCoV may be synergistic and lead to severe tracheobronchitis (An et al., 2010).

There were no significant differences in the sex, age and vaccination status of dogs between single and multiple virus detections. This findings 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 (Cillóniz 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 (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 (Prachayangprecha et al., 2013; Shoji et al., 2011; Yang et al., 2011).

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.

Because of novel emerging viruses, recent literature 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., 2015; 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., 2012), 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., 2015). 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., 2015). 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 problems (Choi et al., 2015; Kapoor et al., 2012).

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., 2009, 2010; Lau et al., 2011, 2012). 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 (Choi et al., 2015; Kapoor et al., 2010, Lau et al., 2012). 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). 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 (Kapoor et al., 2012, Choi et al., 2015). 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 attempted to discover an associated pathogen(s) from dogs suffering from acute respiratory disease that gained negative result from routine respiratory virus detections. Fortunately, the CanineCV genome was discovered from two dogs' lungs (14P105D, 14P112N) suffering from respiratory distress by using NGS. In this study, the CanineCV genome could be detected in lung that was consistent with previous findings (Li et al., 2013). However, the fresh tissues of

CanineCV-infected dogs were limited for intestinal tract, altering the valuable information of viral distribution could not be gathered.

Analysis of nearly complete genome of CanineCV detected in Thailand was conducted. The results 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 isloates 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. Preliminary analysis of CanineCV Thai isolates revealed discordant phylogenetic relationship with other CanineCV strains. For example, the CanineCV TH2016 strains were clustered within CanineCV UCD3-478 when compared by complete genome and Cap gene; however, they were clustered with a new clade away from CanineCV UCD3-478 strain when constructed the phylogenetic tree by Rep gene. Paradoxical results of those phylogenetic trees were implied to possible potential recombination of CanineCV TH2016 strains. According to *Circoviridae* family, potential naturally genetic recombination events were reported in beak and feather virus (Heath et al., 2004) and Torque teno virus (Manni et al., 2002). Moreover, genetic recombination of one mammalian circovirus, the PCV, was evident among PCV2a and –b, and also in many strains of PCV2 (Hesse et al., 2008; Ma et al., 2007). These findings suggested the possibility of natural recombination events occurring in other circoviruses. Here, we predicted the recombination breakpoint occurring at Rep gene, which is overlapping the ORF3 region.

Because of the lack of genomic function of CanineCV, the information from the PCV counterpart might be useful for further discussion in this study. In fact, the Rep gene, encoded by ORF1 and presented 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 (Hesse et al., 2008; Ma et al., 2007; Olvera et al., 2007; Ramos et al., 2013). However, there are some reports demonstrating 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 relying on the recombination event occurred in the Rep gene of CanineCV. 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., 2010); 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., 2010). 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. Further investigations of the ORF3 function will facilitate a clear idea to elucidate the potential roles of CanineCV infectivity and replication.

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. Nevertheless, the genetic study and phylogenic analysis based on only single genome region derived from this study might be conducted to the wrong interpretation because of genetic recombination of the CanineCV. Continuous investigations of the circovirus evolution resulted from either mutation or recombination or both are essential for the in-depth knowledge of CanineCV. Further study should be focused on the function of each region of CanineCV genome, which might contribute better understanding.

VI. Conclusion

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.

VII. References

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Output ที่ได้จากโครงการ

ผลงานวิจัยที่ได้ทำศึกษาครั้งนี้ สามารถดำเนินการได้สำเร็จครบตามวัตถุประสงค์ของโครงการทุกข้อ ซึ่งประกอบไปด้วย

1) สามารถพัฒนาเทคนิคพีซีอาร์แบบซิมเพล็กซ์ และมัลติเพล็กซ์ได้ เพื่อนำมาใช้ในการวินิจฉัยเชื้อไวรัสก่อโรคระบบทางเดินหายใจซับซ้อนในสุนัข โดยมีการเปรียบเทียบค่าความไวและความจำเพาะ

2) สามารถนำเทคนิคดังกล่าวมาใช้ในการเฝ้าระวังโรคไวรัสที่มีการแพร่ทางอากาศในทางระบาดวิทยา ซึ่งอาจมีการแพร่กระจายจากสัตว์สู่คนได้

3) สามารถทราบความชุกและความหลากหลายทางพันธุกรรมของเชื้อไวรัสก่อโรคระบบทางเดินหายใจซับซ้อนในสุนัขที่มีการระบาดอยู่ ณ ปัจจุบัน และเปรียบเทียบกับลักษณะทางพันธุกรรมที่มีผู้รายงานไว้แล้วในฐานข้อมูลสากล

4) ค้นพบไวรัสสายพันธุ์ใหม่ที่ไม่เคยมีรายงานการระบาดมาก่อนในประเทศไทย ได้แก่ Canine bocavirus และ Canine circovirus ซึ่งให้ผลลบต่อการตรวจด้วยเทคนิคพีซีอาร์ที่ได้พัฒนาขึ้นมา โดยเทคนิค NGS

5) ใช้ข้อมูลของรหัสพันธุกรรมของเชื้อไวรัสใหม่ดังกล่าว ในการสำรวจและเฝ้าติดตามความชุกของเชื้อไวรัสดังกล่าวในสุนัขในประเทศไทยต่อไป ซึ่งยังไม่ทราบบทบาทในการก่อโรคอย่างแน่ชัดทั้งในคนและสัตว์

ผลงานวิจัย (Output) ที่ได้รับ

1) ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ

1.1) ที่ได้รับตอบรับตีพิมพ์แล้ว (Published) จำนวน 2 ฉบับ ได้แก่

1.1.1) 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. (Impact factor 0.782 from ISI; 2015) (ภาคผนวก 1)

1.1.2) Piewbang C., Rungsipipat A., Poovorawan Y., **Techangamsuwan S.** 2017. Viral molecular and pathological investigations of *Canid herpesvirus* 1 infection associated respiratory disease and acute death in dogs. Acta Vet.-Beogr. 67(1): 11-24. (Impact factor 0.741 from ISI; 2015) (ภาคผนวก 2)

1.2) ที่ได้ทำการส่งเพื่อพิจารณาตอบรับ (Submitted) จำนวน 1 ฉบับ ได้แก่

1.2.1) Piewbang C., **Techangamsuwan S.**, Rungsipipat A., Poovorawan Y. Cross-sectional investigation and risk factor analysis of community-acquired and hospital-associated canine infectious respiratory disease complex. ในวารสาร PLOS ONE

2) ผล sequencing nucleotide ของเชื้อ Canine bocavirus และ Canine circovirus ซึ่งกำลังอยู่ในระหว่างการนำไปเผยแพร่ในฐานข้อมูลสากล Pubmed (http://www.ncbi.nlm.nih.gov)

3) กิจกรรมอื่นๆที่เกี่ยวข้อง ได้แก่

3.1) ผลงานอื่นๆ เช่น การไปเสนอผลงาน การได้รับเชิญไปเป็นวิทยากร

3.1.1) วิทยากรบรรยายพิเศษ ในงานประชุมวิชาการนานาชาติ The 18th Federation of Asian Veterinary Associations (FAVA) Congress 2014 ณ ประเทศสิงคโปร์ วันที่ 28-30 พฤศจิกายน 2557 ในหัวข้อเรื่อง “Molecular Diagnostic Tool for Multiple Canine Respiratory Viral Infections: Challenge Cases”

3.1.2) นำเสนอผลงานในรูปแบบโปสเตอร์ ในงานประชุมวิชาการนานาชาติทางสัตวแพทย์ของคณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ครั้งที่ 14 (The 14th Chulalongkorn University Veterinary Conference (CUVC2015)) ณ ศูนย์การค้าสยามพารากอน เมื่อวันที่ 20-22 เมษายน 2558 ในหัวข้อเรื่อง “Fatal Systemic Canine Herpesvirus Infection Associated with Acute Death in Neonatal Puppies” และตีพิมพ์ใน Proceeding ของงานประชุมดังกล่าว (ภาคผนวก 3)

3.1.3) นำเสนอผลงานในรูปแบบโปสเตอร์ ในงานประชุมประจำปี “นักวิจัยรุ่นใหม่..พบ..เมธีวิจัยอาวุโส สกว.” ครั้งที่ 15 (TRF-OHEC Annual Congress 2016) เมื่อวันที่ 6-8 มกราคม 2559 ในหัวข้อเรื่อง “Prevalence of Canine Infectious Respiratory Disease Complex associated Viruses in Dogs Using Multiplex PCR in Southern Thailand” และเผยแพร่บทคัดย่อใน Proceeding ของงานประชุมดังกล่าว ในรูปแบบซีดี (ภาคผนวก 4)

3.1.4) วิทยากรบรรยายพิเศษ ในงานประชุมวิชาการนานาชาติทางสัตวแพทย์ของคณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ครั้งที่ 15 (The 15th Chulalongkorn University Veterinary Conference (CUVC2016)) ณ ตึกเฉลิมราชกุมารี (จามจุรี 10) จุฬาลงกรณ์มหาวิทยาลัย เมื่อวันที่ 20-22 เมษายน 2559 ในหัวข้อเรื่อง “Canine Infectious Respiratory Disease Complex Viruses: A Diagnostic Platform, Status and Discovery” และตีพิมพ์ใน Proceeding ของงานประชุมดังกล่าว (ภาคผนวก 5)

3.1.5) นำเสนอผลงานในรูปแบบโปสเตอร์ จำนวน 2 เรื่อง ในงานประชุมวิชาการนานาชาติทางสัตวแพทย์ของคณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ครั้งที่ 16 (The 16th Chulalongkorn University Veterinary Conference (CUVC2017)) ณ ศูนย์ประชุมแห่งชาติสิริกิติ์ เมื่อวันที่ 22-24 มีนาคม 2560 ในหัวข้อเรื่อง “Investigation of Canine Infectious Respiratory Disease Complex in

Thailand during 2013- 2015” และ “Acute Progressive Bronchointerstitial Pneumonia in a Dog: A Case Report” และตีพิมพ์ใน Proceeding ของงานประชุมดังกล่าว (ภาคผนวก 6)

3.1.6) วิทยากรบรรยายพิเศษ ในงานประชุมใหญ่สามัญประจำปี 2560 และการประชุมวิชาการบำบัดโรคสัตว์เลี้ยง ครั้งที่ 23 (The 10th VPAT Regional Veterinary Congress (VRVC2017)) ณ อิมแพ็คฟอรั่ม ศูนย์การแสดงสินค้าและการประชุมอิมแพ็ค เมืองทองานี เมื่อวันที่ 23-26 กรกฎาคม 2560 ในหัวข้อเรื่อง “Advanced diagnostic tools for infectious disease: when, what, where, how?” และตีพิมพ์ใน Proceeding ของงานประชุมดังกล่าว (ภาคผนวก 7)

3.1.7) ให้ความอนุเคราะห์ตรวจวินิจฉัยกรณีศึกษา ในสุนัขที่มีอาการของโรคติดเชื้อระบบทางเดินหายใจ หรือสุนัขที่เสียชีวิตโดยมีรอยโรคที่ปอดแบบเฉียบพลัน แก่นิสิตระดับบัณฑิตศึกษา คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย และโรงพยาบาลสัตว์เอกชน

3.2) การเชื่อมโยงทางวิชาการกับนักวิชาการอื่นๆ ทั้งในและต่างประเทศ

3.2.1) ได้ทำงานวิจัยร่วมกับ อ.สพ.ญ. วิภาพร จารุจารีต คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเทคโนโลยีราชมงคลศรีวิชัย ในการสำรวจและเก็บตัวอย่างสุนัขที่ป่วยด้วยโรคระบบทางเดินหายใจ เพื่อตรวจสอบหาเชื้อไวรัสในกลุ่ม CIRDC ในพื้นที่แทบภาคใต้ ในหัวข้อเรื่อง “ Detection of canine infectious respiratory disease complex associated viruses in dogs with multiplex RT-PCR in the South of Thailand” ซึ่งเป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาสัตวแพทยศาสตรบัณฑิต ของนักศึกษา จำนวน 4 คน ได้แก่ นายธฤต กุลฉันท์วิทย์ นางสาวธนาทิพย์ รัตนสมบูรณ์ นางสาวสุกีนา สะยะมิง และ นางสาววิภาพร จารุจารีต โดยเป็นอาจารย์ที่ปรึกษาร่วม

3.2.2) ได้ทำงานวิจัยร่วมกับ Prof. Dr. Wolfgang Baumgartner จาก Department of Pathology และ Prof. Dr. Albert D.M.E. Osterhaus จาก Research Center for Emerging Infections and Zoonoses, University of Veterinary Medicine, Hannover ประเทศเยอรมันนี ในการศึกษาหาเชื้อไวรัสสายพันธุ์ใหม่ โดยใช้เทคนิค Next-generation sequencing (NGS) ในตัวอย่างของสุนัขป่วย โดยรับนิสิตระดับปริญญาเอกไปทำการศึกษาวิจัยที่ประเทศเยอรมันนี้ เป็นระยะเวลา 7 เดือน