Characterization of canine enteric viruses in Vietnam

ベトナムの犬における腸管ウイルスの性状解析

The United Graduate School of Veterinary Science
Yamaguchi University

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THE UNITED GRADUATE SCHOOL OF VETERINARY SCIENCE

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1. General introduction

1.1. Canine distemper virus

1.1.1. History

Canine distemper, caused by canine distemper virus (CDV), is the highly contagious disease in domestic dogs and has been known since at least 1760. Clinical features of the disease were first described by Edward Jenner in 1809 (James and Lachlan, 2011). In 1905, CDV was first isolated by Henri Carré (Sharon *et al.,* 2000).

Canine distemper has been reported in all famillies of terrestrial carnivores, peccary, seal and monkeys. Currently, at least 14 distinct genetic lineages of CDV are recognized worldwide, based on sequence analysis of the hemagglutinin (H) gene: Asia-1, Asia-2, Asia-3, Asia-4, America-1, America-2, America-1, South America-2 and South America-3 (Zhao *et al.,* 2010; Guo *et al.,* 2013; Radtanakatikanon *et al.,* 2013; Yi *et al.,* 2013; Espinal *et al.,* 2014).

1.1.2. Classification and genome structure

CDV is a negative-sense, single-stranded RNA virus that is a member of the order *Mononegavirales*, family *Paramyxoviridae,* genus *Morbillivirus*, which include a number of highly pathogenic viruses, such as measlse virus, rinderpest virus and *peste-des-petits*-ruminants virus. The genome of CDV encodes six structural proteins, nucleocapsid (N), matrix (M), fusion (F), H, phospho- (P) and large- (L) proteins, and two nonstructural proteins, C and V proteins, that were found as extratranscriptional units within the P gene (Carvalho *et al.,* 2012) (Fig.1.1). The H gene is one of the most variable genes in CDV and has been used to investigate the genetic relationships among the various strains (Gámiz *et al.,* 2011).

1.1.3. Disease and pathogenesis

CDV spreads by aerosol droplets, contacts with epithelium of the upper respiratory tract of dogs and replicates in tissue macrophages within 24 hour. By 2-4 days post infection, the number of virus increase in tonsils and retropharyngeal and bronchial lymph nodes. By 4-6 days post infection, replication of viruses occurs within lymphoid follicles in spleen, stomach and small intestine. By 8-9 days post infection, virus spreads to all epithelia tissues and central nervous system. In dogs with poor immune status, virus spread to many tissues including skin, exocrine and endocrine gland, epithelium of gastrointestinal, respiratory and genitourinary tracts by 9-14 days post infection. Clinical signs in these dogs are dramatic and severe, and virus persists in their tissues until death (Craig, 2006). Clinical signs vary depend on virulence of virus strain, environmental conditions, age and immue status and include vesicular and pustular dermitis in puppies, footpad keratinocytes, neurologic manifestations and hemorrhagic diarrhea (Craig, 2006; Carvalho *et al.,* 2012).

1.1.4. Epidemiology

Viral shedding occurs by 7 days post infection. CDV is commonly transmitted by aerosol or droplet exposure and is isolated from various tissues and secretion. Virus can be excreted up to 60-90 days post infection, although shorter period of shedding are more typical (Craig, 2006). CDV can infect to all species of the families *Canidae* (dog, dingo, fox, coyote, jackal, wolf), *Procyonidae* (raccoon, coatimundi, panda), *Mustelidae* (weasel, ferret, fishers, mink, skunk, badger, marten, otter), the large members of the family *Felidae* (lion, leopard, cheetah, tiger), and the collared peccary (*Tayassu tajacu*) (Appel *et al.,* 1991; Sharon *et al.,* 2000; Kameo *et al.,* 2012) and caused high mortality in black-footed ferret (*Mustela nigripes*), the bat-eared fox

(*Otocyon megalotis*), red pandas (*Ailurus fulgus*), hyenas (genus *Hyaena*), African wild dogs (genus *Lycaon*), raccoons (genus *Procyon*), palm civets (*Paradoxurus hermaphroditus*), and Caspian (*Pusa caspica*) and Baikal seals (*P. sibirica*) (James and Lachlan, 2011, Beineke *et al.,* 2015; Pope *et al.,* 2016). Recently, CD outbreaks have been reported in non-human primate (*Macaca mulatta*) in China and Japan. (Qiu *et al.,* 2011; Sakai *et al.,* 2013).

CDV genotype Asia-1 was first reported in Japan in 1991 (Mochizuki *et al.,* 1999). Since then, this genotype has been reported in many Asian countries, including Taiwan in 2003 (Liang *et al.,* 2008; Chulakasian *et al.,* 2012), Korea in 2007 (An *et al.,* 2008), Thailand in 2013 (Radtanakatikanon *et al.,* 2013) and China in 2004 to 2015 (Zhao *et al.,* 2010; Li *et al.,* 2014; Cheng *et al.,* 2015). Recently, some CD outbreaks by genotype Asia-1 infection had caused deaths of tigers and monkeys (Nagao *et al.,* 2012; Sakai *et al.,* 2013). CDV genotype Asia-2 was also found in Japan, Korea and Thailand (Lan *et al.,* 2007; An *et al.,* 2008; Sultan *et al.,* 2009). Recently, CDV genotypes Asia-3 and Asia-4 were detected in China (Zhao *et al.,* 2010; Bi *et al.,* 2015). The other CDV genotypes have also been reported in many countries (Martella *et al.,* 2006; Demeter *et al.,* 2007; Gámiz *et al.,* 2011). In Vietnam, CDV genotype America-1 was reported in domestic dogs in 2009 (Lan *et al.,* 2009).

1.1.5. Diagnosis, prevention and control

Diagnosis of canine distemper is based on characteristic clinical signs in practice. However, laboratory diagnosis is necessary to exclude the other diseases with similar clinical signs. Many laboratory tests are available for diagnosis of CDV infection such as immunohistochemistry, RT-PCR and virus isolation (Frisk *et al.,* 1999; Craig, 2006). RT-PCR tests can be carried out from conjunctival and rectal

swabs, blood mononuclear cells, any tissue sample with epithelium, feces and urine. Further, virus isolation using canine signaling lymphocytic activation molecule (SLAM)-expressing cells is also performed to confirm the suspicious CDV infection or investigation of biological characterization of field viruses (Tatsuo *et al.,* 2001; Lan *et al.,* 2007; Nakano *et al.,* 2009; Kameo *et al.,* 2012).

Control of CDV infection is based on adequate diagnosis, quarantine, sanitation, and vaccination. CDV is sensitive to many disinfectants against virus (Watanebe *et al.,* 1989; Sanekata *et al.,* 2010). Since CDV-infected animals or their secretion are sources of the virus, they should be separated from CDV-susceptible animals. Immunization of pups with vaccine is the best method for prevention of CDV infection. Modified live vaccines can be used in puppies at 6 weeks old and then repeat at 2 to 4 week intervals until 16 weeks old, which is the standard protocol (James and Lachlan, 2011). Some cases of canine distemper occur in vaccinated puppies, raising the obvious question whether the signs are caused by the vaccine virus or a field strain. Recently, it is reported that new CDV strain causing CD outbreak in domestic dogs in America might escape from efficiency of available vaccines (Riley *et al.,* 2015).

1.2. Canine coronavirus (CCoV)

1.2.1. History

CCoV was first described during an epizootic in a canine military unit in Germany in 1971 (Binn *et al.,* 1974). Since then, several CCoV outbreaks have been reported worldwide, showing that CCoV is an important enteropathogen in the dog. CCoV was divided into two genotypes, CCoV-I and -II, belonged to genus *Alphacoronavirus*, species *Alphacoronavirus-1* and is similar to feline coronavirus

(FCoV) type I and type II (Buonavoglia *et al.,* 2006) and transmissible gastroenteritis virus (TGEV). CCoV was further divided into two subgenotype IIa and IIb based on *N*-terminal domain of Spike (S) protein (Pratelli *et al.,* 2006; Poder *et al.,* 2011; Licitra *et al.,* 2014a).

1.2.2. Classification and genome structure

CCoV is a single-stranded, positive-sense RNA virus and belongs to order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae*, genus *Alphacoronavirus*, species *Alphacoronavirus-1*. The first two-thirds of the coronavirus genome consists of two overlapping open reading frames (ORFs) that encode non-structural proteins including the viral RNA-dependent RNA polymerase and proteases. Last one-third of the genome encodes the major structural S, envelope (E), membrane (M), and nucleocapsid (N) proteins **(**Decaro and Buonavoglia, 2008) (Fig.1.2). The S protein has an important role in binding to the host cell receptor and triggers fusion between viral and cellular membranes (Licitra *et al.,* 2014a). In addition, CoV encodes small nonstructural proteins, ORFs 3a, 3b, 3c, located between S and E genes and ORFs 7a, 7b, located between the N gene and poly A. The function of these nonstructural proteins still remains unknown (Poder *et al.,* 2011).

1.2.3. Disease and pathogenesis

The natural transmission is faecal-oral route and virus in faeces is the major source of infection. After ingestion, CCoV enter into the mature epithelial cells of the villi of small intestine (Craig, 2006). In neonatal puppies, the virus appears to replicate in the villus tips of the enterocytes of the small intestine, causing a lytic infection followed by desquamation and shortening of the villi. Malabsorption and

deficiency of digestive enzymes follow, resulting in diarrhea which occurred by 18–72 hours post infection. In some severe cases, diarrhea can become watery, dehydration and electrolyte. Most of affected dogs recover spontaneously 8-10 days after onset of symptoms (Pratelli, 2006).

1.2.4. Epidemiology

CCoV has been isolated in several outbreaks of enteritis in dogs. Neonatal puppies are affected more severely than weanings and adults. Viral shedding is in the faeces for 6-9 days post infection, but some infected dogs have shed virus for weeks or months or longer (Pratelli, 2006). CCoV infection in domestic dogs have been reported in many countries, including Japan, China, Brazil, United States (U.S), European countries (Soma *et al.,* 2011; Costa *et al.,* 2014; Licitra *et al.,* 2014b; Decaro *et al.,* 2010). Currently, there is no data of CCoV infection in domestic dogs in Vietnam.

1.2.5. Diagnosis, prevention and control

Virus neutralization (VN) test and ELISA (Enzyme-linked immunosorbent assay) can be performed to detect antibody against CCoV antibody (Bandai *et al*., 1999; Pratelli *et al*., 2002). Detection of CCoV in fecal samples can be carried out by electron microscope, isolation in cell cultures and RT-PCR. Recently, real-time RT-PCR with Taqman probe has been developed for detection and quantification of CCoV-I and CCoV-II in fecal samples (Pratelli *et al.,* 2004; Decaro *et al.,* 2010).

CCoV is highly contagious and once the virus has become established in the environment, control of CCoV infection is difficult. To prevent the transmission of virus, healthy animals should be separated from infected dogs and their excretions.

Crowding, unsanitary conditions, stress and the other conditions enhance development of clinical disease. The significance of CCoV vaccines for protection of dogs under field conditions is controversial (Pratelli, 2006). Inactivated and modified live virus vaccine are available for protection against CCoV infection, but provide incomplete protection in elimination of viral replication (Pratelli *et al.,* 2003; Craig, 2006). Evaluation of CCoV vaccine efficacy against disease is difficult because CCoV infection show no clinical signs or mild sign (Craig, 2006).

1.3. Canine parvovirus

1.3.1. History

Minute virus of canines was first isolated from canine fecal specimens in 1967, which is now called canine parvovirus 1 (CPV-1). Canine parvovirus 2 (CPV-2) was first described as a new disease in 1978 and recognized as highly virulent pathogen throughout the world. There is no antigenic relationship between CPV-1 and -2 (Mochizuki *et al.,* 2002; James and Lachlan, 2011). CPV-2 is believed to be originated as a host range variant of feline panleukopenia virus (FPLV) that has been know since the 1920s to infect cats, mink and the other animals (Truyen, 2006). In 1980s, CPV-2 was replaced by the new virus type that could be distinguished using monoclonal antibodies. This new antigenic type was termed as CPV-2a and then other type, CPV-2b, emerged and quickly spread worldwide. In 2000, a new genotype, CPV-2c, was recognized in Italy (Bounavoglia *et al.,* 2001), it has recently been reported in many countries in Europe, Asia and U.S (Decaro *et al.,* 2007; Hong *et al.,* 2007; Decaro *et al.,* 2012).

1.3.2. Classification and genome structure

The family *Parvoviridae* includes two subfamilies *Parvovirinae* and *Densovirinae.* The subfamily *Parvovirinae* are divided into five genera, *Protoparvovirus, Erythrovirus, Dependovirus, Amdovirus* and *Bocavirus.* CPV-2 belongs to genus *Protoparvovirus* which includes FPLV, mink enteritis virus (MEV) and raccoon parvovirus (RPV) (Decaro *et al.,* 2012). The genome of CPV-2 is a single-stranded linear DNA and encodes three structural protein (VP1, VP2 and VP3) and two nonstructural proteins (NS1 and NS2) (Fig.1.3). The VP2 capsid protein is a major determinant of antigenicity and host range of CPVs (Phromnoi *et al.,* 2010). CPV-1 belonged to genus *Bocavirus* and genetically and is antigenically unrelated with CPV-2 (Mochizuki *et al.,* 2002; Tattersall *et al.,* 2005).

1.3.3. Disease and pathogenesis

CPV-2 are transmitted from dogs to dogs by oronasal exposure by containminated feces. After virus entry in the oropharynx, initial virus replication occurs in pharyngeal lymphoid tissue, mensenteric lympho nodes and thymus. Plasma viremia occurred 1-5 days after infection. Subsequent to verimia, virus localizes mainly in the gastrointestinal epithelium, oral and esophageal mucosa, lymphoid tissue such as thymus, lympho nodes and bone marrow and small intestine. Virus may be also found in the lungs, spleen, liver, kidney and myocardium (Craig, 2006). Virus attack to villus of intestinal membrance and crypt cells lead to maldigestion and malabsorption, with resultant diarrhea (James and Lachlan, 2011). The incubation period is 3-7 days after infection, following clinical signs, vomiting, hemorrhagic diarrhea, losses of appetite, depression, fever and dehydration in puppies. Myocarditis may be observed in neonatal puppies (Miranda and Thompson, 2016).

1.3.4. Epidemiology

CPV-2 is high contagious and transmitted to sensitive dogs by contact with contaminated feces. People, veterinary instruments, rodents and insects may be machinery vectors in virus infection. CPV-2 infection can been observed in dogs of any age, sex and breed. However, puppies between 6 weeks and 6 months old, and Rottweilers, Labrador retrievers, Doberman pinshers, American Staffordshire terriers, Alaskan sled dogs and German shepherds have been reported with a high risk for CPV enteritis (Craig, 2006; Miranda and Thompson, 2016). CPV-2 variants (2a, 2b, 2c) have been reported at least in 37 countries for CPV-2a, 31 countries for CPV-2b and 21 countries for CPV-2c (Mairanda and Thompson, 2016). In Asia countries, CPV infection have been reported in Korea, India, Thailand, Taiwan, Japan, and China (Yoon *et al.,* 2009; Kumar and Nandi, 2010; Phromnoi *et al.,* 2010; Chou *et al.,* 2013; Soma *et al.,* 2013; Lin *et al.,* 2014). In Vietnam in 2002, CPV-2b was predominant in domestic dogs (Nakamura *et al.,* 2004).

1.3.5. Diagnosis, control and prevention

CPV infection has been diagnosed based on clinical signs such as foul smelling and bloody diarrhea in young dogs (age under 2 years old). Since all dogs with bloody diarrhea are not infected with CPV, the other enteropathogic bacterial infection should be also examined (Craig, 2006). Laboratory tests are also used for confirmation of CPV infection. Since CPV has hemagglutination (HA) activity to erythorocytes, hemagglutination-inhibition (HI) test is also used for detection of antibody against CPV. Virus-neutralization test and ELISA is also used to detect CPV-specific antibody (Ikeda *et al.,* 1998; Litster *et al.,* 2012). Recently, many molecular methods were developed for detection of CPV in feces such as PCR and

real-time PCR (Buonavoglia *et al.,* 2001; Chakravarti *et al.,* 2016). Electron microscope scan can detect virus in feces or tissue and virus isolation is often used for further research of CPV. Some kits for detection of CPV antigen in feces were also available in veterinary hospital (Gagnon *et al.,* 2016; Miranda and Thompson, 2016).

The major method for control and prevention of CPV infection is vaccination. Attenuated and inactivated vaccine are available. These CPV vaccines are inoculated in combination with the other vaccine components (Craig, 2006). The evolution of CPV-2 raise questions about the efficacy of some vaccines which are originated from old CPV-2 strains. The vaccines originated from old strains might be inefficient for protection against new field genotypes (Truyen, 2006). Recently, CPV-2b vaccine have been developed for control of CPV infection (Wilson *et al.,* 2014). However, the cross-protection of licensed CPV-2 and CP-V2b vaccines against CPV-2c in puppies should be further investigated (Hernández *et al.,* 2015).

1.4. Canine kobuvirus

1.4.1. History

Canine kobuvirus (CKoV) is believed to have been originated from Aichi virus 20-50 years ago. Aichi virus was first described in humans with gastroenteritis in the Aichi Prefecture, Japan, in 1989 (Yamashita *et al.,* 1991). CKoV is one of Aichivirus A and has only one genotype. In dogs, CKoV was detected in outbreaks of acute gastroenteritis in canine shelters in U.S (Kapoor *et al.,* 2011). CKoV has been detected in diarrheic dogs (Li *et al.,* 2016).

1.4.2. Classification and genome structure

CKoV is a single strand (ss) RNA (+) virus and belongs to genus *Kobuvirus* in the family *Picornaviridae*. Genus *Kobuvirus* is divided into three species, *Aichivirus*

A, *Aichivirus B* (formerly Bovine kobuvirus) and *Aichivirus C* (formerly Porcine kobuvirus*)*. CKoV belongs to *Aichivirus A* and is considered to be a genotype (CaKV type 1) distinct from murine kobuvirus (MuKV type 1) and human AiV (AiV type 1) (Martino *et al.,* 2013). CKoV has one large open reading frame (ORF) encoding a single polyprotein that is cleaved into 3 structural capsid proteins (VP0, VP1, and VP3) and 7 non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D) (Kapoor *et al.,* 2011) (Fig.1.4).

1.4.3. Diseases and pathogenesis

Diseases caused by CKoV infection in domestic dogs have been still remains unclear. CKoV has never been isolated from domestic dogs, and detection of CKoV RNA showed that this virus was not a major cause of diarrhea in dogs (Vicente *et al.,* 2013). Aichi virus is a causative agent of acute gastroenteritis in humans and causes gastroenteritis, diarrhea, abdominal pain, nausea, vomiting and fever (Yamashita *et al.,* 1991). A previous report showed that porcine kobuvirus seemed to be associated with diarrhea in pigs (Park *et al.,* 2013). Bovine kobuvirus have been detected in cattle, but any disease associated with this virus is not confirmed (Reuter *et al.,* 2011).

1.4.4. Epidemiology

CKoV has been recognized in several countries in domestic dogs and wild animals. Prevalence of CKoV in domestic dogs has been reported to be 2.34% in Italy, 17.9% in China, 1.25% in UK and 19% and 13.2% of diarrheic and health dogs in Korea, respectively (Martino *et al.,* 2013; Vicente *et al.,* 2013; Oem *et al.,* 2014; Castillo *et al.,* 2015; Li *et al.,* 2016)**.** In Japan, 37.2% of diarrheic dogs and 48% of

clinically healthy kenneled dogs were positive for CKoV (Soma *et al.,* 2016). In Vietnam, there is no information on CKoV infection in domestic dogs.

1.4.5. Diagnosis, control and prevention

CKoV is very difficult to be isolated that there is no isolate from dogs. Diagnosis of CKoV infection is mainly based on molecular methods. RT-PCR have been developed for detection of CKoV in feces (Vicente *et al.,* 2013; Choi *et al.,* 2014; Li *et al.,* 2016). The role of CKoV as a pathogen in domestic dogs remain unclear. Further investigation will be required for clarification of the pathogenesis. Currently, there is no vaccine for kobuviruses, including Aichi virus. Disinfection should be helpful for decrease of transmission of kobuvirus. Howerver, kobuvirus is resistant to many disinfectants (alcohols, chlorine, chloroform), some potential disinfectants such as acetic acid, glutaraldehyde, sodium hydroxide and Virkon-S®11 might be effective to Kobuvirus (Proietto *et al.,* 2016).

1.5. Mammalian orthoreovirus

1.5.1. History

Mammalian reoviruses (MRVs) were first discovered as ‘‘respiratory and enteric orphans’’ in the U.S and Mexico in the early 1950s. Reoviruses were isolated from healthy humans without any clinical symptom of enteric or respiratory disease (Day *et al.,* 2009). In 1961, MRV was isolated from domestic dogs with clinical signs in U.S. (foamy saliva, vomiting and bloody diarrhea) (Lou *et al.,* 1963). In 1979, MRV was successfully isolated from healthy dogs in Japan (Murakami *et al.,* 1979). Isolation of MRV from diarrheic dogs was reported in Italy (Decaro *et al.,* 2005).

Recently, MRV were divided into four serotypes, including serotype 1 (Lang), serotype 2 (D5/Jones), serotype 3 (Dearing) and serotype 4 (Ndelle) by VN and HI tests (Sabin *et al.,* 1959; Knipe, 2013).

1.5.2. Classification and genome structure

MRV is a double-stranded (ds) RNA virus and belongs to Genus *Orthoreovirus* in the family *Reoviridae.* MRV contains 10 genome segments, which are named as large (L1, L2 and L3), medium (M1, M2 and M3) and small (S1, S2, S3 and S4) genes based on their electrophoretic mobility (Nibert *et al.,* 1990) (Fig.1.5). S1 gene of MRVs encodes σ1 protein which is located on the outer capsid of the virion and responsible for viral attachment to cellular receptors. Therefore, σ1 protein is a major factor for determination of serotype (Decaro *et al.,* 2005).

1.5.3. Disease and pathogenesis

MRV often transmits at fecal-oral route and the infection is systemic, often appearing as necrosis and inflammatory in several organs (James and Lachlan, 2011). Several MRV strains caused acute gastroenteritis and encephalitis in human (Steyer *et al.,* 2013; Lelli *et al.,* 2015). In domestic dogs, respiratory symptoms (nasal discharge, foamy saliva and cough) and fever (39.5^{o}C - 40^{o}C) were observed after experimental infection (Lou *et al.,* 1963). An experimental intranasal infection of mice with MRV-3, which isolated from masked palm civet, resulted in fatal respiratory distress, tissue damage and inflammation, and high virus titer in the lungs (Li *et al.,* 2015).

1.5.4. Epidemiology

Nonfusogenic MRV can infect to many species, including pigs, cattle and dogs (Knipe., 2013). MRV can be isolated from dogs with respiratory, enteric symptoms or healthy dogs in U.S, Japan and Italy (Lou *et al.,* 1963; Murakami *et al.,* 1979; Kobubu *et al.,* 1993, Decaro *et al.,* 2005). MRV might co-infect with other agents to enhance their disease (Buonavoglia *et al.,* 2007). Four dogs inoculated MRV-1, which was isolated from sick dog, developed signs of illness related to respiratory system (Lou *et al.,* 1963). However, the other MRV-1 strain IU41, which was isolated from healthy dog, did not cause any illness to dogs (Murakami *et al.,* 1979). MRV could cause mild pathogenesis in both wild and domestic cats after experimental infection (Knipe, 2013). Seroprevelance of MRVs in domestic dogs in Japan showed that prevalence of T1L (Lang), T2A (Amy) and T3D (Dearing) were 53.9%, 33.9% and 46.2%, respectively (Hwang *et al.,* 2013). In Vietnam, there is no available information on MRV infection in domestic dogs.

1.5.5. Diagnosis, control and prevention

Reovirus infections can be diagnosed by virus isolation and RT-PCR from many kind of clinical samples from patients or infected animals such as tissue, throat swabs, urine, stool, and cerebral spinal fluid (Knipe, 2013). For determination of viral serotypes, HI and plaque-reduction neutralization tests were carried by using type-specific antisera. These methods can also be used for detection and titlation of antibody in infected dogs (Hwang *et al.,* 2013). Nucleotide sequence of S1 gene was also determined for viral serotyping. In domestic dogs, RT-PCR can be performed from fecal sample (Leary *et al.,* 2002; Decaro *et al.,* 2005). Since co-infection between MRV and other agents might lead to serious disease (Buonavoglia *et al.,*

2007), disinfection of excretion and segregation of infected dogs should be considered for prevention. No vaccine is available for dogs.

1.6. The aim of this study

Although there are a few reports of canine enteric viruses in Vietnam, the information on these viruses is limited. In this study, we investigated the enteric virus infections in domestic dogs with and without diarrhea and carried out genetic analysis to understand their characteristics. In addition, virus isolation was also performed to analyze biological characteristics of viruses and to develop new vaccines in Vietnam in future.

1.7. Figures

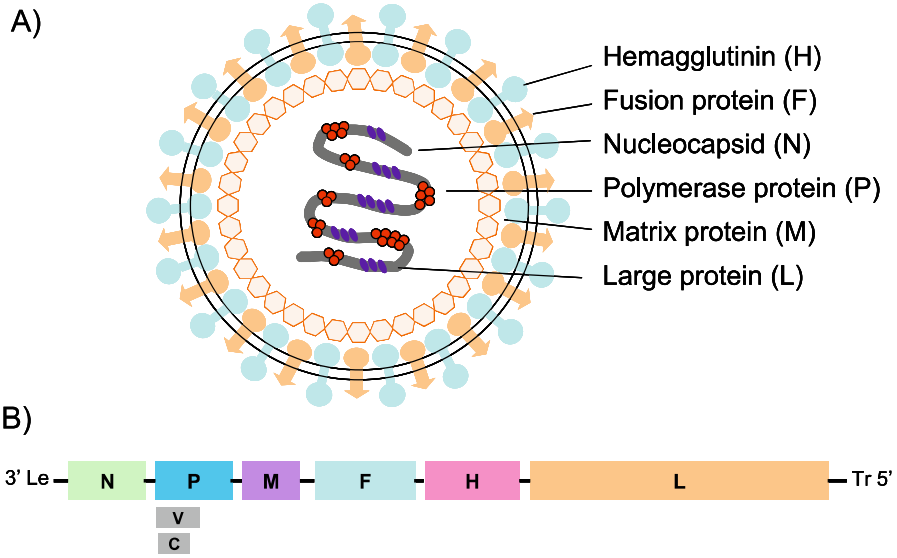


Fig.1.1. Structure (A) and genomic organization (B) of canine distemper virus

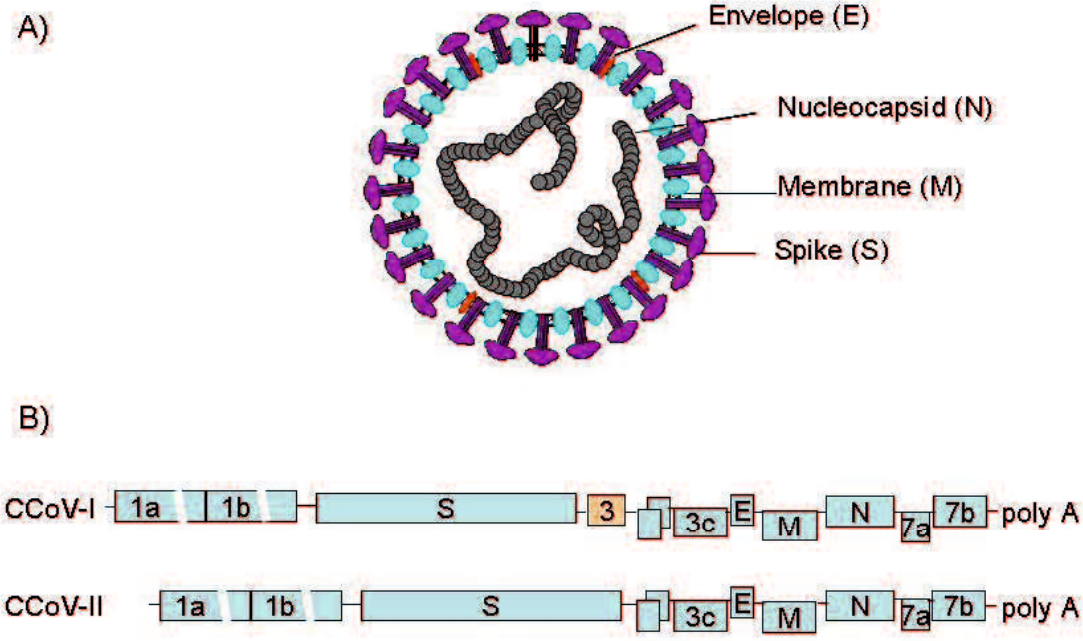


Fig.1.2. Structure (A) and genomic organization (B) of canine coronavirus

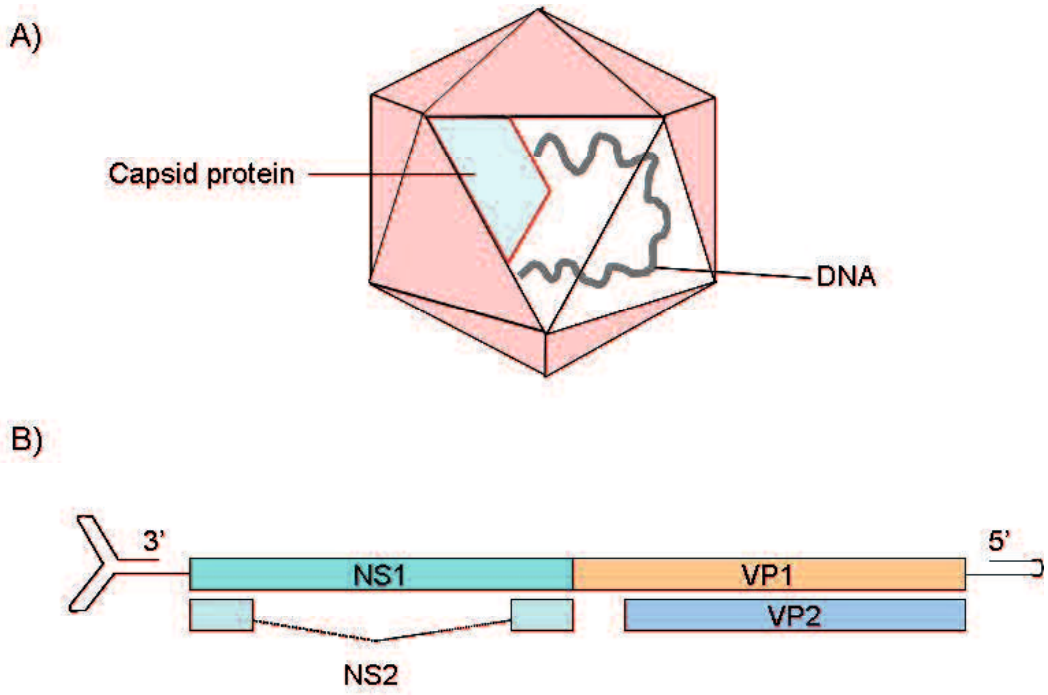


Fig.1.3. Structure (A) and genomic organization (B) of canine parvovirus

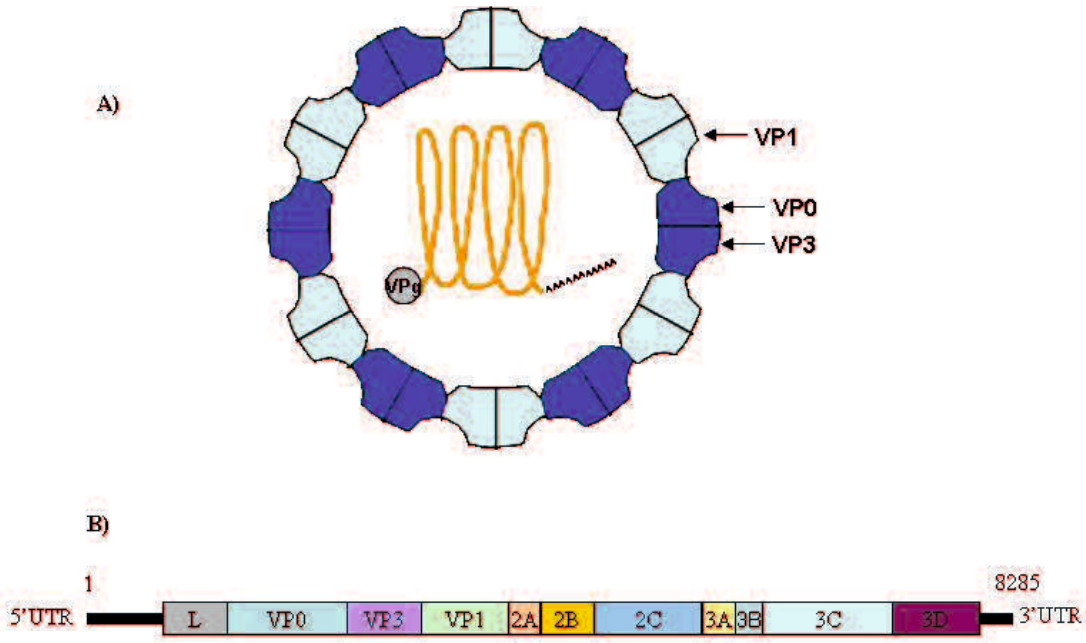


Fig.1.4. Structure (A) and genomic organization (B) of canine kobuvirus

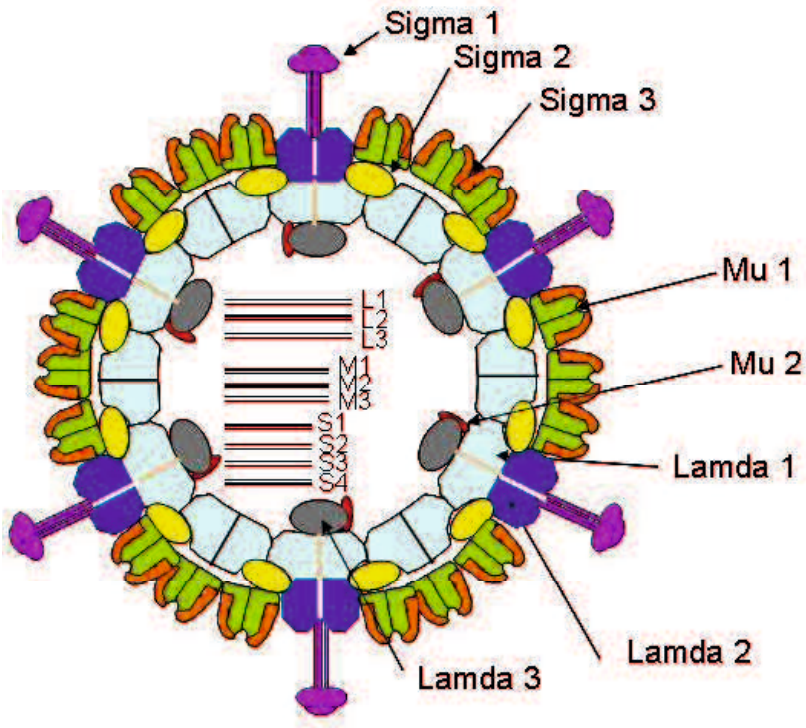


Fig.1.5. Structure and genomic organization of mammalian orthoreovirus

2. Chapter 1

Isolation and phylogenetic analysis of canine distemper virus among domestic dogs in Vietnam

2.1. Abstract

CDV is one of the most serious pathogens found in many species of carnivores, including domestic dogs. In this study, H genes were detected in five domestic Vietnamese dogs with diarrhea and two CDVs were successfully isolated from dogs positive for H genes. The complete genome of one isolate, CDV/dog/HCM/33/140816, was determined. Phylogenetic analysis showed that all Vietnamese CDVs belonged to the Asia-1 genotype. In addition, the H proteins of Vietnamese CDV strains were the most homologous to those of Chinese CDVs (98.4% to 99.3% identity). These results indicated that the Asia-1 genotype of CDV was the predominant genotype circulating among the domestic dog population in Vietnam and that transboundary transmission of CDV has occurred between Vietnam and China.

2.2. Introduction

CDV is an infectious agent that can cause canine distemper (CD), a lethal disease, among many species of carnivores (Apple *et al.,* 1994; Appel *et al.,* 1995; Harder *et al.,* 1996; Beineke *et al.,* 2009). Recently, a CDV epidemic was reported among non-human primates in Chinese and Japanese quarantines (Sakai *et al.,* 2008; Qiu *et al.,* 2011). CDV is a negative-sense, single-stranded RNA virus that is a member of the order *Mononegavirales*, family *Paramyxoviridae,* genus *Morbillivirus.* The genome of CDV encodes six structural proteins (N, M, F, H, P and L proteins) and two nonstructural proteins (C and V proteins) (Carvalho *et al.,* 2012). The H gene is one of the most variable genes in CDV and has been used to investigate the genetic relationships among the various strains (Gámiz *et al.,* 2011). At least 14 different genotypes of CDV have been reported, including Asia-1, Asia-2, Asia-3, Asia-4, Europe, European wildlife, Arctic-like, Rockborn-like, America-1, America-2, Africa, South America-1, South America-2 and South America-3 (Zhao *et al.,* 2010; Radtanakatikanon *et al.,* 2013; Yi *et al.,* 2013; Espinal *et al.,* 2014; Guo *et al.,* 2013). Although there has been one report of the isolation of the genotype America-1 from Vietnamese dogs in Ha Noi, Vietnam (Lan *et al.,* 2009), information on CDVs in Vietnam is very scarce.

In this study, CDVs were detected and isolated from Vietnamese dogs with clinical symptoms and were genetically analyzed.

2.3. Materials and Methods

2.3.1. Collection of fecal and eye swab samples

A total of 43 fecal swabs and 10 eye swabs were collected from diarrheic dogs in one veterinary hospital in Ho Chi Minh, Vietnam, from 2013 to 2014. Their ages

ranged from 2 months to 3 years. The swab samples were dissolved in 2 ml of phosphate-buffered saline and were then filtered through a 0.22-µm filter (Millipore, Carrigtwohill, Ireland) and stored at -80°C until analysis.

2.3.2. Viral RNA extraction

RNA was extracted from CDV-infected A72/SLAM cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and from the filtered fecal and eye swab samples using the Viral RNA Mini Kit (Qiagen).

2.3.3. Reverse transcription polymerase chain reaction (RT-PCR) for H genes

To determine the H genes of the CDVs, RT-PCR was performed using QIAGEN One Step RT-PCR (Qiagen) with the primers HF (5'-AAC TTA GGG CTC AGG TAG TC-3') and HR (5'-AGA TGG ACC TCA GGG TAT AG-3') (Demeter *et al.,* 2007). The amplified products were confirmed to be approximately 2 kbp in size by electrophoresis on 0.8% agarose gels, and were purified using the QIAquick PCR Purification Kit (Qiagen).

2.3.4. Sequence analysis

The nucleotide sequences were determined using an ABI PRISM 310 Genetic Analyzer auto sequencer (Applied Biosystems, Carlsbad, CA, USA). The full length of the H gene (1,824 bp) was determined as described previously (Kameo *et al.,* 2012).

2.3.5. Virus isolation

For virus isolation, A72 cells expressing canine SLAM, A72/cSLAM (Nakano *et al.,* 2009), were used. The cells were maintained in Dulbecco's minimum essential

medium (DMEM; Life Technologies, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 100 units/ml penicillin and 100 µg/ml streptomycin (Life Technologies). A72/cSLAM cells in 6-well plates were inoculated with the filtrates of the swab samples and then incubated at 37°C in a 5% CO_2 incubator. Cells were observed daily for a cytopathic effect (CPE). If no CPE was present, the cells were blind-passaged five times. The isolates were plaque-purified three times using Crandell-Rees feline kidney cells expressing canine signaling lymphocyte activation molecule (CRFK/cSLAM) (Nakano *et al.,* 2009).

2.3.6. Nucleotide sequence of the complete genome of CDV/dog/HCM/33/140816

To determine the nucleotide sequence of the complete genome of Vietnamese CDV, one representative isolate, CDV/dog/HCM/33/140816, was analyzed. The TaKaRa RNA LA PCR™ Kit (AMV) Ver. 1.1 (Takara, Otsu, Japan) was used to amplify the whole genome of this CDV strain with the following primer pairs: 53F, 5'-CTT AGG GTC AAT GAT CCT ACC-3', and 4977R, 5'-TGG AGG GGA TCT TGT AGG GT-3' (4,925 bp); 4398F, 5'-GTT ATC ATC AGC GAT GA TCAG -3', and 9076R, 5'-AGA TGG ACC TCA GGG TAT AG-3' (4,679 bp); 6947F, 5'-AAC TTA GGG CTC AGG TAG TC-3', and 12457R, 5'- GGT TCC TTA ATG CTC TCG C-3' (5,511 bp); and 11832F, 5'-GCA CCC ATA GGT GGT CTT AAT-3', and 15523R, 5'- GTC TCA AGT TGA AAG AGC CAA TTC-3' (3,692 bp). The PCR products were sequenced using specific primers, which were designed according to the overlapping strategy. The 5'- and 3'-terminal sequences were determined using the 5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen,

Waltham, CA, USA). The sequence was assembled and analyzed using GENETYX® ver. 8 (Software Development Co., Tokyo, Japan).

2.3.7. Phylogenetic analysis

Phylogenetic relationships based on the amino acid sequences of the H proteins and the nucleotide sequence of the genome or each gene were analyzed using a distance-based (neighbor-joining) method with MEGA 6.0 software (Tamura *et al.,* 2013). Bootstrap values were calculated based on 1,000 replicates.

2.4. Results

2.4.1. Detection and isolation of CDV in Vietnamese dogs

A total of five CDV strains were amplified by the RT-PCR for H genes. All dogs from which CDVs were detected had diarrhea and were less than 1 year old (Table 2.1). The Vietnamese CDVs were named CDV/dog/HCM/01/131018, CDV/dog/HCM/24/140112, CDV/dog/HCM/29/140127, CDV/dog/HCM/33/140816 and CDV/dog/HCM/38/140827. These CDV-positive samples were inoculated into A72/cSLAM cells for virus isolation. Two CDVs, CDV/dog/HCM/33/140816 and CDV/dog/HCM/38/140827, were isolated.

2.4.2. Comparison of Vietnamese CDVs with other CDVs

Five H genes (1,824 bp, 607 amino acids) were determined and the nucleotide sequences were deposited into the DNA database of Japan (DDBJ) (Accession numbers: LC159583, LC159584, LC159585, LC159586 and LC159587). All Vietnamese CDV strains possessed G at position 530 (530G) and Y at position 549 (549Y) in the H proteins. A total of nine potential asparagine-linked glycosylation

sites were conserved. The percent identity between the amino acids of the H protein among the Vietnamese CDVs ranged from 98.5% to 100%; between Vietnamese CDVs and genotype Asia-1, it ranged from 96.5% to 99.5%; between Vietnamese CDVs and genotype Asia-2, it ranged from 92.9% to 93.6%; between Vietnamese CDVs and genotype Asia-3, it ranged from 86.2% to 86.7%; between Vietnamese CDVs and genotype Asia-4, it ranged from 93.7% to 94.8%; between Vietnamese CDVs and genotype America-1, it ranged from 89.6% to 91.4%; between Vietnamese CDVs and genotype America-2, it ranged from 93.9% to 96.2%; between Vietnamese CDVs and genotype Europe, it ranged from 93.4% to 95.7%; between Vietnamese CDVs and genotype European wildlife, it ranged from 94.1% to 95.4%; between Vietnamese CDVs and genotype Artic, it ranged from 92.3% to 94.1%; between Vietnamese CDVs and genotype Rockborn-like, it ranged from 94.6% to 95.9%; between Vietnamese CDVs and genotype South America, it ranged from 93.2% to 94.9%; and between Vietnamese CDVs and genotype Africa, it ranged from 93.9% to 94.7%.

2.4.3. Complete sequence of a Vietnamese CDV

CDV/dog/HCM/33/140816 was isolated from the eye swabs of dogs with diarrhea and was plaque-purified three times. The complete genome was determined and deposited into the DDBJ (accession number: LC159587). The genome of the CDV/dog/HCM/140816 strain is 15,690 bases long and consists of six structure genes in the order of 3′-N-P-M-F-H-L-5′. The open reading frames (ORF) of N, P, M, F, H and L were located at nucleotides 108 to 1,679, 1,081 to 3,324, 3,432 to 4,439, 4,935 to 4,623, 7,079 to 8,902 and 9,030 to 15,584, respectively. The percent identities between the complete sequences of CDV/dog/HCM/33/140816 and 32 other CDVs

deposited in GeneBank ranged from 92.4% to 98.7% and that with the Hebei strain showed the highest sequence similarity (Table 2.2). Phylogenetic trees were constructed based on H gene (Fig.2.1) and the whole genome (Fig.2.2). The results showed that the Vietnamese CDV/dog/HCM/33/140816 was close to Chinese CDVs, Hebei, SY, PS and HLJ1-06.

2.5. Discussion

Although CD has been recognized since the 1950s and vaccines against CDV have been available for controlling the virus, CDV remains a threat to many species of animals. CD outbreaks in both domestic and wild animals have been reported in many countries (An *et al.,* 2008; Liang *et al.,* 2008; Chulakasian *et al.,* 2010; Zhao *et al.,* 2010; Qiu *et al.,* 2011; Nagao *et al.,* 2012; Guo *et al.,* 2013; Yi *et al.,* 2013; Espinal *et al.,* 2014; Li *et al.,* 2014; Cheng *et al.,* 2015). CDVs have been categorized into many genotypes based on the H protein, and the genotypes were consistent with the geographic distribution of the viruses (Mochizuki *et al.,* 1999; Martella *et al.,* 2006). Therefore, the genotypes make it clear where the animals were infected with CDV. In Vietnam, only genotype America-1 had been detected in domestic dogs in 2009 (Lan *et al.,* 2009). In this study, all of the five Vietnamese CDVs identified belonged to genotype Asia-1, indicating that this genotype is dominant among the domestic dog population in Vietnam as in other Asian countries. This is the first report of the isolation of genotype Asia-1 in Vietnam. Genotype Asia-1 of CDV was first reported in Japan in 1991 (Mochizuki *et al.,* 1999). Since then, this genotype has spread to many Asian countries, including Taiwan in 2003 (Liang *et al.,* 2008; Chulakasian *et al.,* 2010), Korea in 2007 (An *et al.,* 2008), Thailand in 2013 (Radtanakatikanon *et al.,* 2013) and China in 2004 to 2015 (Zhao *et al.,* 2010; Li *et al.,* 2014; Cheng *et al.,*

2015). Recently, some CD outbreaks of genotype Asia-1 had caused the deaths of tigers and monkeys in Japan (Sakai *et al.,* 2008; Nagao *et al.,* 2012).

Phylogenetic analysis revealed that all Vietnamese CDVs in this study are closely related to the Hebei (Mink, 2008), SY (Raccoon dog, 2012), PS (Dog, 2010) and HLJ1-06 (Fox, 2006) strains found in China (Fig.2.1, Fig.2.2, Fig.2.3). These results suggested that there might be cross-border transmission of CDV between China and Vietnam.

Another genotype of CDV, America-1, has also been reported to have caused CD outbreaks in Vietnam (Lan *et al.,* 2009). However, the Vietnamese CDVs analyzed in the present study possessed low identity with genotype America-1 (89.6% to 91.4% identity in the amino acid sequences of H genes). Therefore, a vaccine against CDV genotype America-1 might not completely protect domestic dogs from CDV infection in Vietnam. Further investigations will be required to clarify the efficacy of a Vietnamese vaccine.

In this study, two Vietnamese CDV strains were successfully isolated and their whole genomes were analyzed. Further investigations of the biological characteristics of Vietnamese CDVs would be useful for the diagnosis of CD and for vaccine development in Vietnam.

2.6. Conclusion

In conclusion, our results showed that the Asia-1 genotype of CDV was the predominant genotype circulating among the domestic dog population in Vietnam, and transboundary transmission of CDV has occurred between Vietnam and China.

2.7. Figures, legends and tables

Fig.2.1. Phylogenetic tree based on the amino acid sequences of the H gene (607 amino acids).

The Vietnamese CDV strains isolated in this and previous studies are shown in bold. Genbank accession numbers are shown in parentheses. The accession numbers of reference strains in collapsed branches (triangles) are as follows. European wildlife: H06Ny11 (ABK35780), H06Ny13 (ABK35782), Danish Mink (CAA87688) and German ferret (CAA59358). Asia-4: NJ(11)2 (AHN52227), NJ(12)4 (AHN52229) and NJ(12)5 (AHN52230). Rockborn: Rockborn (ADN96002), Rockborn Candur (ADU4476) and lesser panda (AAD54601). Europe: 5804 (AAQ49703), Dog Turkey (AAM11476), DK91A (AAQ05829) and 2544 (CAB01252). America-2: 16407 (ACD92997), 171391-513 (AHM26181.1), A75/17 (AAD49703.1), 01-2689 (AAT94553.1), Black panther A-92 (CAA90879) and America dog (CAA87691). Africa: 4L70214 (ACS36478), 21L (ACS36479) and 1bn (ACS36498). Arctic: H05Bp7F (ABK35776), H05Bp10S (ABK35779), H06Bp2S (ABK35771), CDV2784/2013 (AHF81428.1), Greenlandic dog (CAA87689) and PDV2 (CAA59357). Asia-2: 50Con (BAN16508.1), 50Con (BAN16508.1), 55L (BAN16489.1), 5L (BAN16489.1), 55L (BAN16489.1), 55L (BAN16489.1), 26D (BAB39166), 98-002 (BAA84208) and HM3 (BAB39167). The nucleotide sequences of Haku00 and Haku06 were reported by Hirama *et al.* (2004). Scale bar indicates the number of amino acid substitutions per site.

Fig.2.2. Phylogenetic tree based on the nucleotide sequences of the CDV genomes.

The Vietnamese CDV strain, CDV/dog/HCM/33/140816, isolated in this study is shown in bold. GenBank accession numbers are shown in parentheses.

Fig. 2.3. Phylogenetic analysis based on the nucleotide sequences of N, P, M, F and L genes and the amino acids of C and V proteins. The Vietnamese strain, CDV/dog/HCM/33/140816, isolated in this study is shown in red.

Table 2.1. Epidemiological information of Vietnamese dogs from which CDV were isolated or detected by RT-PCR

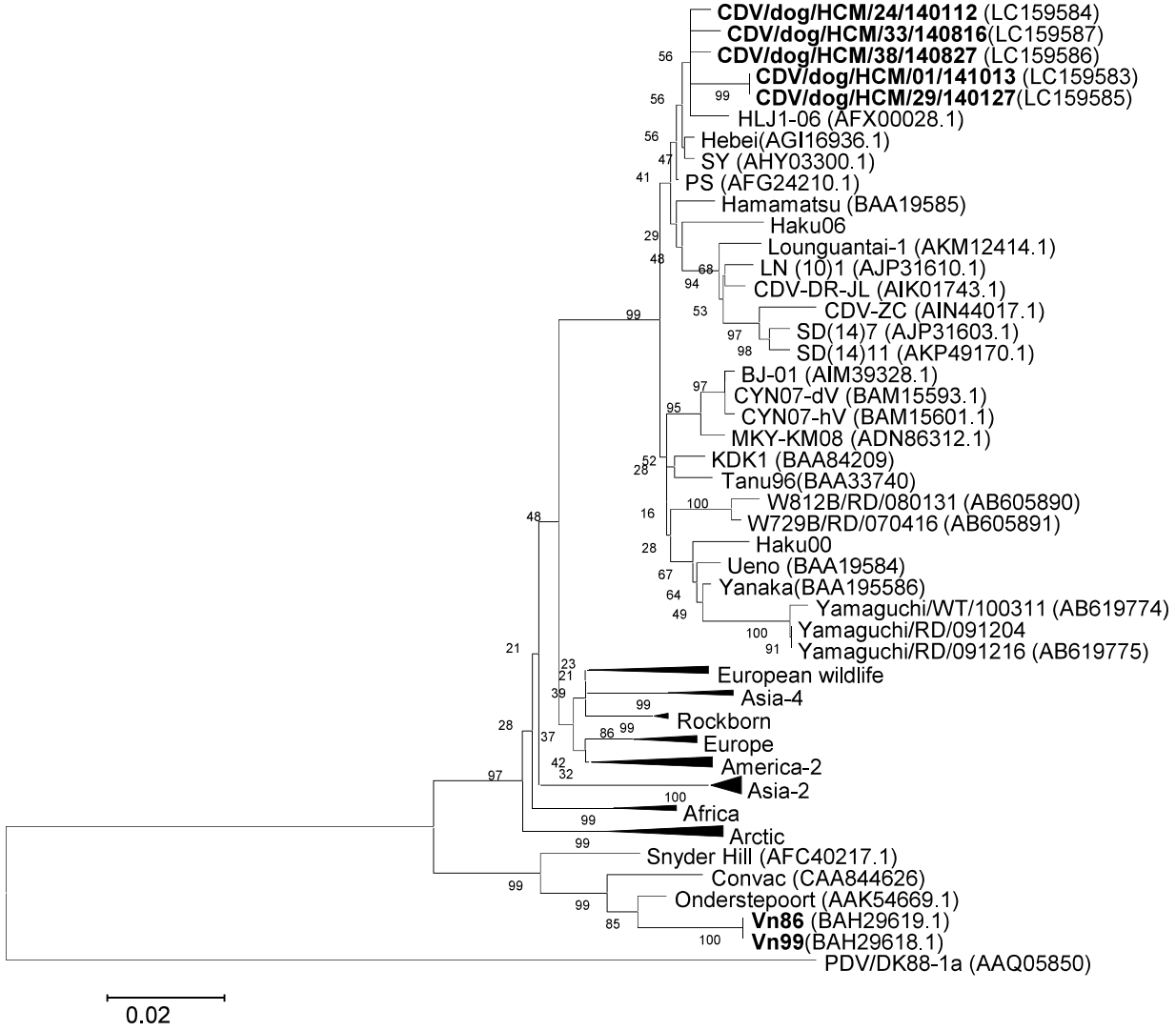
Strains	Breeds	Place	Age (month)	Sex	Date	Clinical sign	RT-PCR or isolation
CDV/dog/HCM/01/141013	Vietnamese dog	Thu Duc	8	Male	18/10/2013	Diarrhea, cough, nasal discharge	RT-PCR (feces)
CDV/dog/HCM/24/140112	Vietnamese dog	District 6	4	Female	12/01/2014	Diarrhea	RT-PCR (feces)
CDV/dog/HCM/29/140127	Vietnamese dog	District 10	4	Male	27/01/2014	Diarrhea	RT-PCR (feces)
CDV/dog/HCM/33/140816	Vietnamese dog	Binh Chanh	5	Female	16/08/2014	Diarrhea	Isolation (eye swab)
CDV/dog/HCM/38/140827	Vietnamese dog	Binh Tan	2	Female	27/08/2014	Cough, diarrhea	Isolation (feces, eye swab)

Table 2.2. Identities of nucleotide sequence of CDV/dog/HCM/33/140816 with those of the other strains

Strain/genotype	Accession number	Place	Host	Identity (%)								
				Genome	5'-NCR*	N	P	M	F	H	L	3'-NCR*
Asia-1												
Hebei	KC427278.1	China	Mink/2008	**98.7**	**98.1**	**99.1**	**98.4**	**98.9**	**98.5**	**98.9**	**98.7**	**99.1**
HLJ1-06	JX681125.1	China	Fox/2006	**98.2**	**97.2**	**98.9**	**99.1**	**99.1**	**98.3**	**98.8**	**97.6**	**99.1**
PS	JN896331.1	China	Dog/2010	**98.6**	94.4	**99.4**	**99.1**	**99.1**	**98.2**	**98.7**	**98.6**	92.5
CYN07-hV	AB687721.2	Japan	Monkey/2008	**97.3**	**99.1**	**98.0**	**97.7**	**98.3**	**96.6**	**97.2**	**97.5**	94.3
SD(14)7	KP765763.1	China	Fox/2014	**97.7**	**98.1**	**98.3**	**98.3**	**99.0**	**96.8**	**97.2**	**98.1**	94.3
SY	KJ466106.1	China	Raccoon dog	**98.5**	**99.1**	**99.0**	**98.8**	**98.6**	**97.8**	**98.7**	**98.7**	**99.1**
Lounguantai-1	KP677502.1	China	Panda/2015	**97.8**	**98.1**	**97.8**	**98.1**	**98.4**	**97.4**	**97.4**	**98.1**	94.3
SD(14)11	KP738610.1	China	Raccoon dog/2014	**97.7**	**98.1**	**98.3**	**98.1**	**99.0**	**97.0**	**97.1**	**98.0**	94.3
BJ-01	KF856711.1	China	Monkey/2006	**97.3**	**97.2**	**98.0**	**97.6**	**98.3**	**96.6**	**97.1**	**97.4**	94.3
MKY-KM08	HM852904.1	China	Raccoon dog/2008	**97.2**	**97.2**	**97.6**	**97.8**	**98.2**	**96.3**	**97.1**	**97.4**	92.5
LN(10)1	KP765764.1	China	Fox/2010	**98.0**	**98.1**	**98.5**	**98.2**	**98.9**	**97.3**	**97.7**	**98.2**	**96.2**
CYN07-dV	AB687720.2	Japan	Monkey/2008	**97.3**	**99.1**	**98.0**	**97.7**	**98.3**	**96.6**	**97.2**	**97.5**	94.3
CDV-ZC	KJ994343.1	China	Raccoon dog/2013	**96.8**	94.4	**97.6**	**98.1**	**98.4**	**94.8**	**97.0**	**97.1**	**95.3**
CDV-DR-JL	KJ848781.1	China	Raccoon dog/2014	**98.0**	**97.2**	**98.6**	**98.4**	**98.6**	**97.2**	**97.9**	**98.2**	94.3
Asia-2												
50Con	AB476402.1	Japan	Dog/2009	94.3	94.4	**95.7**	**95.1**	**96.1**	92.6	92.5	**95.0**	90.6
007Lm	AB490680.1	Japan	Dog/2009	94.2	94.4	**96.1**	94.9	**96.1**	92.2	92.5	**95.0**	93.4
011C	AB476401.1	Japan	Dog/2009	94.1	94.4	**95.7**	**95.0**	**96.1**	92.2	92.5	94.9	90.6
55L	AB475099.1	Japan	Dog/2009	94.2	94.4	**95.7**	94.9	**96.1**	92.2	92.4	**95.0**	90.6
M25CR	AB475097.1	Japan	Dog/2009	94.1	94.4	**95.7**	**95.0**	**96.0**	92.2	92.5	94.9	90.6
009L/H	AB490672	Japan	Dog/2010	94.1	94.4	**95.7**	94.9	**96.1**	92.1	92.4	**95.0**	90.6
Europe												
5804	AY386315.1	Germany	Dog/2003	**95.2**	93.5	**97.1**	**96.3**	**96.1**	93.9	**95.0**	**95.5**	88.7
Arctic												
CDV2784/2013	KF914669.1	Italy	Dog/2013	93.8	89.7	**95.8**	**95.3**	**95.4**	92.2	92.8	94.6	86.8
America-1												
Onderstepoort	AF378705	USA	Dog/2001	92.4	93.5	94.0	93.6	94.2	90.8	91.4	93.5	88.7
Snyder_Hill	JN896987.1	USA	Dog/2012	92.5	96.3	93.7	93.8	94.6	90.7	91.1	93.5	86.8
CDV-L	KM926612.1	China	fitchew/1992	92.4	96.3	93.4	93.8	94.1	90.9	91.2	93.5	86.8
CDV3	EU726268.1	China	Mink	92.4	95.3	93.4	93.7	93.8	90.9	91.2	93.4	86.8
Shuskiy	HM063009.1	Kazakstan	Mink/2007	92.4	96.3	93.5	93.8	94.2	91.0	91.1	93.5	86.8
Phoca/Caspian/2007	HM046486	Kazakstan	Seal/2007	92.4	96.3	93.4	93.7	94.1	90.9	91.1	93.5	86.8
America-2												
A75/15	AF164967	Switzeland	Ferret/1999	**95.9**	94.4	**97.8**	**96.6**	**97.4**	94.2	**95.2**	**96.3**	93.4
01-2689	AY649446.1	USA	Raccoon/2004	**95.2**	94.4	**97.9**	**96.3**	**97.3**	93.6	93.4	**95.8**	92.5
164071	EU716337.1	USA	Dog/2004	**95.7**	94.4	**98.0**	**96.1**	**97.3**	93.8	94.7	**96.3**	93.4
171391-513	KJ123771.1	USA	Dog/2004	**95.2**	94.4	**97.1**	**96.1**	**97.3**	93.6	94.4	**95.6**	93.4

* NCR : non-coding region

Fig.2.1


CDV/dog/HCM/24/140112 (LC159584)
CDV/dog/HCM/33/140816(LC159587)
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CDV/dog/HCM/01/141013 (LC159583)
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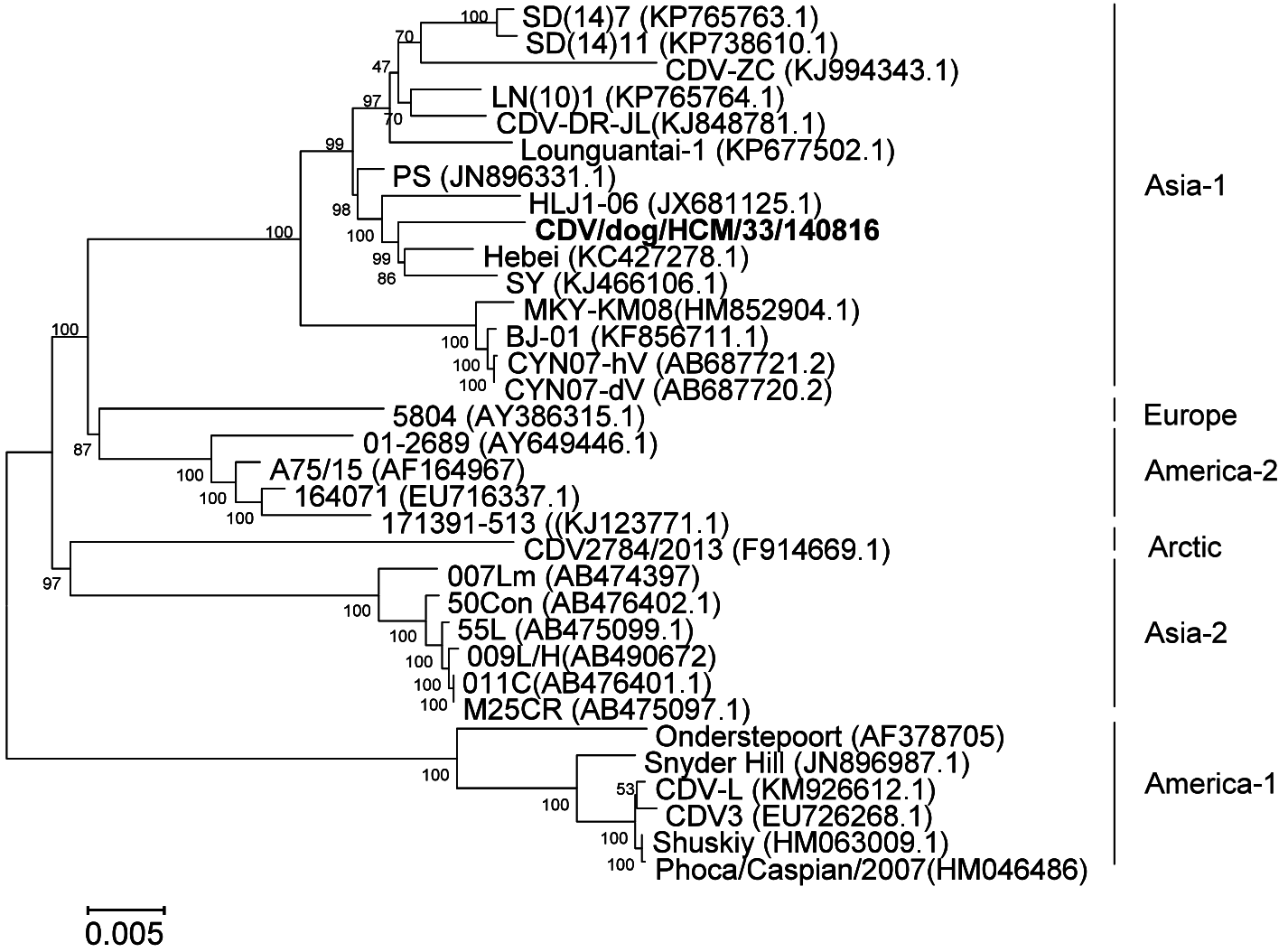
Fig.2.2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Shuskiy (HM063009.1)
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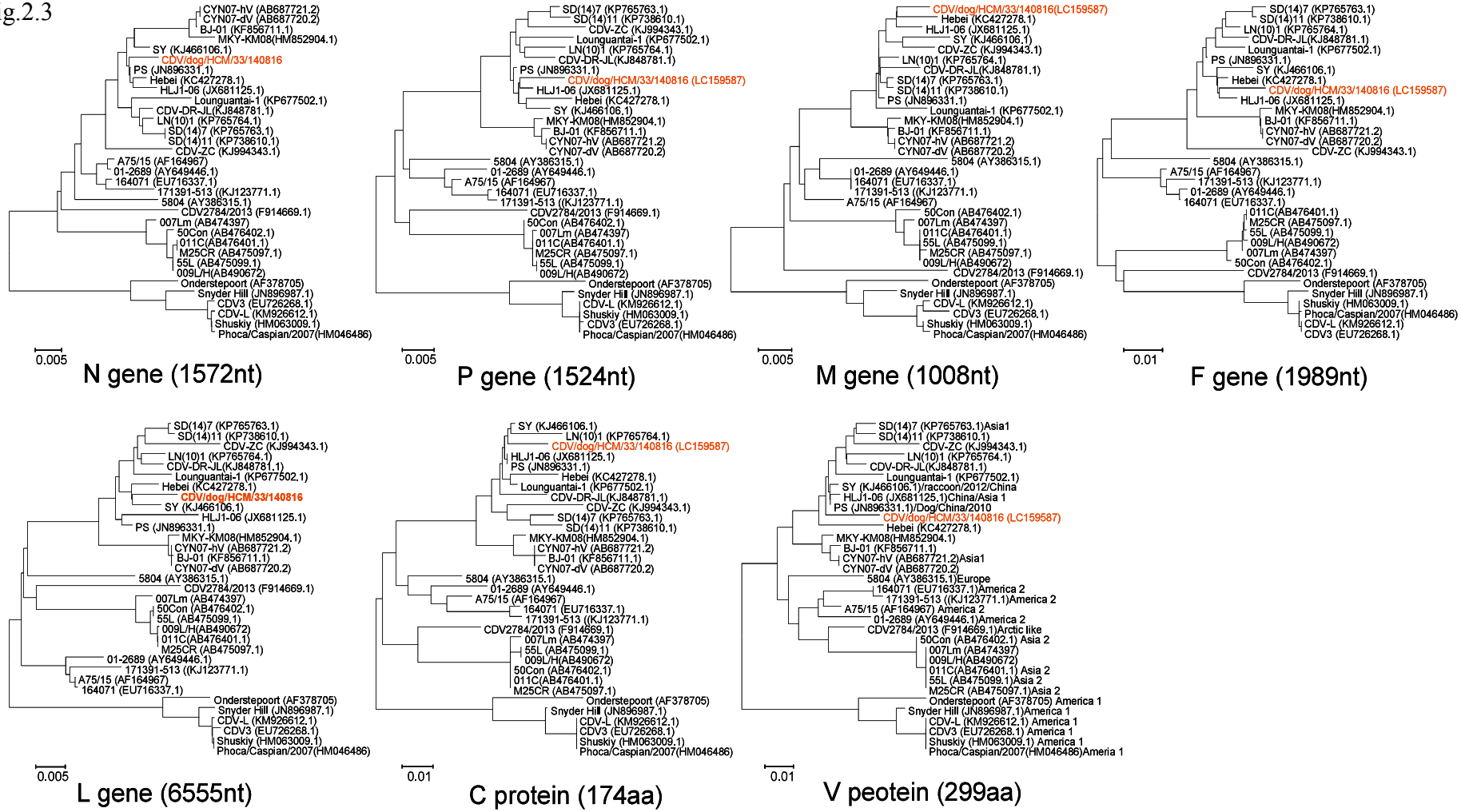
Fig.2.3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Phoca/Caspian/2007(HM046486)Ameria 1
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V peotein (299aa)

3. Chapter 2

Characterization of canine coronavirus spread among domestic dogs in Vietnam

3.1. Abstract

CCoV is an important pathogen that causes enteritis in dogs, but there is no information on CCoV infection in Vietnam. To examine the prevalence of CCoV infection among Vietnamese dogs, 201 serum samples were analyzed by VN test. The results showed that antibody against CCoV-II was present in 87 dogs (43.3%). To detect genes of CCoV, fecal samples collected from 30 diarrheic and 50 healthy dogs were examinated by RT-PCR, confirming that 2 diarrheic dogs and 5 healthy dogs were positive for CCoV. Nucleotide sequences of *N*-terminal region of S gene indicated that CCoV strains were divided into two subgenotypes, CCoV-IIa and IIb, respectively. Furthemore, we succeeded in isolating CCoV/dog/HCM47/2015, the isolate was plaque-purified three times, and 3'-terminal one-third of the genome was analyzed. Interestingly, the plaque-purified virus had a large deletion in ORF3abc and E genes (1,165 nt), and a short deletion in ORF7b gene (60 nt), suggesting that these regions are not necessary for *in vitro* replication of CCoV. Next, the antigenicity between the isolated CCoV-IIb and the other CCoV-IIa was compared by VN test, revealing that antigenicty of the isolated CCoV is equal or higher than that of the other CCoV. In summary, two subgenotypes of CCoV-II are spreading among Vietnamese dogs. The isolated virus with a large deletion after *in vitro* passage may be useful for the development of vaccine, owing to its antigenicity and efficient viral growth *in vitro*.

3.2. Introduction

CCoV was first recognized in an outbreak of gastroenteritis among dogs in 1971 (Binn *et al.,* 1974). Since then, CCoV infection has been reported in dogs in many regions (Sakulwira *et al.,* 2003; Wang *et al.,* 2006; Gur *et al.,* 2008; Decaro *et al.,* 2010; Soma *et al.,* 2011; Costa *et al.,* 2014; Jeoung *et al.,* 2014; Licitra *et al.,* 2014b; Takano *et al.,* 2015). To date, however, information on CCoV in Vietnam has been unavailable.

CCoV is a single-stranded, positive-sense RNA virus that belongs to the genus *Alphacoronavirus*. CCoV was divided into two genotypes, CCoV type I (CCoV-I) and CCoV type II (CCoV-II), which can be differentiated by nucleotide sequences of the spike (S) gene and the presence of ORF3 located downstream of the S gene (Decaro *et al.,* 2008; Pratelli *et al.,* 2011). CCoV-II is also divided into two subgenotypes, CCoV-IIa and CCoV-IIb, based on the amino acid sequence of the *N*-terminal region of the S protein. In the *N*-terminal region of the S protein of CCoV-IIb, the amino acid sequences are highly similar to those of TGEV (Pratelli *et al.,* 2006; Poder *et al.,* 2011; Licitra *et al.,* 2014a).

In this study, the prevalence of CCoV infection among domestic dogs in Vietnam was examined, and Vietnamese CCoVs were genetically and antigenically characterized.

3.3. Materials and Methods

3.3.1. Fecal swabs

A total of 80 fecal swabs were collected from 30 diarrheic and 50 healthy dogs in animal hospitals and households, respectively, in Ho Chi Minh City in Vietnam from 2013 to 2015. Their ages ranged from 2 months to 13 years. Swab

samples were dissolved in 2 ml of phosphate-buffer saline (PBS), filtrated through a 0.22-µm filter (Millipore, Carrigtwohill, Ireland) and stored at -80^{o}C until analysis.

3.3.2. Sera

A total of 201 serum samples were collected from healthy dogs in different districts in Ho Chi Minh City from 2013 to 2015 and stored at -20°C until analysis.

3.3.3. Viral RNA extraction

RNA was extracted from CCoV-infected cells and filtrated fecal swabs using RNeasy Mini kit (Qiagen, Hilden, Germany) and Viral RNA Mini Kit (Qiagen), respectively.

3.3.4. Reverse transciption-polymerase chain reaction (RT-PCR)

RT-PCR was performed using QIAGEN One step RT-PCR (Qiagen). The primer sets ORF3F, 5'-CAC TAA ACT CAA AAT GTT GAT TC-3' and ORF3R, 5'-TTA AGG ATT AAA AAC ATA TTC TA-3' (Poder *et al.,* 2013) and 2bF, 5'-AGG TTG TTG TGG ATG CAT AG-3' and 2bR, 5'-ACG GTC AAG TTC GTC AAG TA-3' (Benetka *et al.,* 2004), were used for detection of CCoV-I and CCoV-II, respectively. Amplified products were confirmed as 628 bp for CCoV-I and 232 bp for CCoV-II by electrophoresis on 2% agarose gels and were then purified using QIAquick PCR Purification kits (Qiagen) for sequencing.

For differentiation of CCoV subgenotypes, primers 1bF, 5'-TTG ATT CAA AGA TTT GAG TAT TGG-3' and CCVSR, 5'-GTT AGT TTG TCT AAT AAT ACC AAC ACC-3', were used to amplify the 5'-terminal region of the S gene (Terada *et al.,* 2014). RT-PCR was performed using a TaKaRa RNA LA PCR ^{TM} kit (AMV) Ver.1.1

(Takara, Otsu, Japan). RT was carried out with random 9-mer primers at 30ºC for 10 min 42ºC for 30 min and 99ºC for 5 min, and then PCR was performed. Amplified fragments were purified using a QIAquick PCR Purification kit (Qiagen) for sequencing.

3.3.5. Virus isolation

Felis catus whole fetus-4 cells (fcwf-4 cells; ATCC Number: CRL-2787) (Jacobse-Geels *et al.,* 1983) were maintained in Dulbecco's minimum essential medium (DMEM; Life Technologies, St. Louis, MO, USA) containing 10% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO, USA), 100 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies). Fcwf-4 cells in 6-well plates were inoculated with extracts from CCoV-positive feces and were then incubated at 37ºC in a 5% CO_2 incubator. Cells were observed daily for cytopathic effects (CPE). If there were no CPE, cells were blind-passaged five times.

3.3.6. Plaque-purification

Isolated CCoV was plaque-purified three times. Briefly, 10 PFU of CCoV were inoculated on fcwf-4 cells in 6-well plates. After 1 hr of adsorption, inoculum was removed, and 0.8% agarose in DMEM containing 10% FCS was overlaid on cells. After the appearance of CPE, plaques were picked up by tips and dissolved into DMEM. This plaque-purification procedure was repeated three times.

3.3.7. Nucleotide sequence analysis

The sequence of the 3´-terminal one-third of the viral genome from S gene to poly A was determined by RT-PCR using TaKaRa RNA LA PCR^{TM} kit (AMV)

Ver.1.1 (Takara). RT was carried out with random 9-mer primers at 30ºC for 10 min, 42ºC for 30 min, 99ºC for 5 min and 4ºC or oligo dT-Adaptor primer at 42ºC for 30 min, 99ºC for 5 min and 4ºC for 5 min. Then, PCR was performed with the following primers: 1bF, 5'-TTG ATT CAA AGA TTT GAG TAT TGG-3'; CCVSR, 5'-GTT AGT TTG TCT AAT AAT ACC AAC ACC-3'; CCVScenF, 5'-TAA GTA ACA TCA CAC TAC C-3'; S6, 5'-CCA AGG CCA TTT TAC ATA AG-3'; ScenFF3, 5'-CTG GAC TGT ACC TGA ATT G-3'; and the reverse primer M13 primer M4, 5'-GTT TTC CCA GTC ACG AC-3'.

In order to amplify the S gene of CCoV-IIa, PCR was performed using the following pimers: 1bF, CCVSR, CCVS2CenF, 5'- CTA TTC TGT GAC ACC ATG TG-3' (Terada *et al.,* 2014) and 2bR. Amplified products were confirmed by electrophoresis on 0.8% agarose gels and were then purified using the QIAquick PCR Purification kit (Qiagen). The nucleotide sequences were determined by an ABI PRISM 310 Genetic Analyzer auto sequencer (Applied Biosystems, Carlsbad, CA, USA). Sequences were assembled and analyzed using GENETYX® ver.8 (Software Development Co., Tokyo, Japan).

3.3.8. VN test

VN testing was performed using fcwf-4 cells as described previously (Shiba *et al.,* 2007; Terada *et al.,* 2014) with modifications. CCoV-IIa fc1, isolated in Japan in 1990 (Bandai *et al.,* 1999), and CCoV-IIb CCoV/dog/HCM47/2015 strain, which was isolated and plaque-purified three times in this study, were used. Briefly, dog sera were inactivated at 56°C for 30 min before VN test. Equal volumes of two-fold serially diluted sera and virus solution containing 2.0×10^3 PFU/ml of CCoV were mixed and incubated at 37°C for 1 hr. Then, 50 μl of this mixture was inoculated onto

fcwf-4 cells monolayer in 24-well plates. After adsorption at 37°C for 1 hr, inoculum was removed, and 0.8% agarose (Lonza, Rockland, ME, USA) in DMEM containing 10% FCS was overlaid. Infected cells were incubated at 37°C until CPE was observed, followed by fixing with phosphate-buffered formalin and staining with crystal violet. When the number of plaques was less than 75% of those in control wells, diluted sera were judged to be positive. Titers were expressed as the highest serum dilution showing 75% plaque reduction or more.

3.3.9. Phylogenetic and statistical analysis

Phylogenetic relationships based on the nucleotide sequences of the 5'-terminal region of S genes or complete S genes were analyzed using distance-based (neighbor-joining) with MEGA 7.0 software (Kumar *et al.,* 2016). Bootstrap values were calculated based on 1,000 replicates.

Chi-squared and Fisher's exact probability tests were used for statistical analysis. *P* values of <0.05 were considered to be statistically significant.

3.4. Results

3.4.1. Prevalence of antibody against CCoV-II in dogs in Ho Chi Minh

Eighty-seven dogs (43.3%) were positive for anti-CCoV-II antibody. Prevalence of anti-CCoV-II antibody in dogs aged over 3 years (52.0%) was significantly higher than that in dogs aged under 1 year (28.6%) (*P<0.05*). Prevalence of anti-CCoV-II antibody in Vietnamese (39.2%) was significantly lower than that in other breeds (63.3%) (Table 3.1). Antibody titer against CCoV-II ranged from 1:10 to 1:320 (data not shown).

3.4.2. Detection of CCoV in diarrheic and healthy dogs

To detect CCoV-I and CCoV-II genes in fecal samples, RT-PCR was carried out using fecal samples from 30 diarrheic and 50 healthy dogs. Although CCoV-I was not detected, CCoV-II was detected from two diarrheic dogs (6.7%) and five healthy dogs (10%, 5/50) (Table 3.2). Next, nucleotide sequences of 5'-terminal region of the S gene were analyzed to differentiate subgenotypes of CCoV-II and were deposited into the DNA database of Japan (DDBJ; Accession No. LC190901-LC190907) (Table 3.2). Phylogenetic analysis based on the 5'-terminal region of the S gene showed that one and six strains belonged to CCoV-IIa and CCoV-IIb, respectively (Fig.3.1 and Table 3.2). Vietnamese CCoVs-IIb formed one cluster and were similar to Chinese ferret badger DM95/2003 (95.4%-95.6%) and CCoV/NTU336/F/2008 (95.2%-95.6%). Vietnamese CCoV-IIa was similar to Raccoon dog GZ43/2003 (95.6%) (Fig.3.1).

3.4.3. Phylogenetic analysis based on S genes

Next, the full length of the S gene of CCoV/dog/HCM27/2014 was also determined (4,362 nt, DDBJ Accession No. LC190906). Phylogenetic analysis based on complete S genes showed that Vietnamese CCoV/HCM47/2015 was similar to Chinese ferret badger CoV/DM95/2003 (95.9%) and CCoV/NTU336/F/2008 (95.2%), and that Vietnamese CCoV-IIa, CCoV/dog/HCM27/2014 was similar to Raccoon dog CoV/GZ43/2003 (97.4%) (Fig.3.2).

3.4.4. Virus isolation and large deletion in plaque-purified CCoV

One CCoV-IIb was successfully isolated from a healthy dog (female, six years old) and designated as CCoV/dog/HCM47/2015. For further analysis, the isolate was plaque-purified three times, and the nucleotide sequence of the 3'-terminal one-third

CCoV genome from the S gene to poly A (8,995 nt) was determined**.** Surprisingly, the plaque-purified CCoV had a large deletion in the ORF3abc and partial E genes (1,165 nt), and a short deletion in the ORF7b gene (60 nt). Next, we determined nucleotide sequences of the original virus in the feces, confirming that the original virus in feces did not have any deletions (DDBJ Accession No. LC190907) (Fig.3.3).

3.4.5. Comparison of antinenicity between CCoV-IIa and IIb

To compare antibody titers against CCoV-IIa and IIb, 60 sera were examined by VN testing using fc1 and plaque-purified CCoV/dog/HCM47/2015, respectively. The results showed that positivity of antibody against CCoV-IIb (58.3%) was slightly higher than that against CCoV-IIa (51.7%) (Table 3.3). Four additional dogs became positive for anti-CCoV-IIb antibody, and nine dogs had significantly higher antibody titers (over 4-fold) against CCoV-IIb than CCoV-IIa (data not shown).

3.5. Discussion

In this study, the prevalence of anti-CCoV-II anibody was 43.3%, suggesting that CCoV-II has been spreading among the dog population in Ho Chi Minh, Vietnam. Seroprevalance of CCoV infection was 15.8% in the open population and 40.8% in kenneled populations in Australia, 44.1% in Japan, 90.8% in Italy and 96.5% in Turkey (Bandai *et al.,* 1999; Pratelli *et al.,* 2002; Gur *et al.,* 2008). Sera from dogs in Vietnam reacted more strongly with CCoV-IIb than CCoV-IIa (Table 3.3), indicating that CCoV-IIb is the major genotype in Vietnam. The results of RT-PCR in this study also supported the notion that CCoV-IIb is the predominant genotype circulating in domestic dogs in Vietnam (Fig.3.1 and Table 3.2).

The prevalance of antibody against CCoV-II increased with age (Table 3.1). Similar results were reported in Turkey (Gur *et al.,* 2008). Older dogs must have more opportunities to be infected with CCoV. Seroprevalence of CCoV-II in imported breeds (63.3%) was significantly higher than that in domestic breeds (39.2%). There have been similar reports in Japan (Soma *et al.,* 2012; Takano *et al.,* 2015), indicating that many dogs are infected with CCoV while housed with breeders or in pet shops.

In this study, two diarrheic dogs (6.7%) and five healthy dogs (10%) were positive for CCoV-II, but no dogs were positive for CCoV-I. One of seven Vietnamese CCoVs strains was positive for CCoV-IIa, and others were positive for CCoV-IIb. Positivity for CCoV-I and CCoV-II in feces of dogs has been reported in Japan (CCoV-I: 53.2 % and CCoV-II: 56.9 %) (Soma *et al.,* 2011)**,** China (CCoV-I: 4.5 % and CCoV-II: 23.9 %) (Wang *et al.,* 2006), Italy (CCoV-I: 25.2 % and CCoV-II: 36.3 %)**,** United Kingdom (U.K) (CCoV-I: 15.0 % and CCoV-II: 20.6 %), Hungary (CCoV-I: 22 % and CCoV-II: 15%), Greece (CCoV-I: 37 % and CCoV-II: 36 %) (Decaro *et al.,* 2010), Brazil (CCoV-I: 47% and CCoV-II: 63%) (Costa *et al.,* 2014) and Korea (CCoV-I: 22 % and CCoV-II: 10 %) (Jeoung *et al.,* 2014). CCoV infection without differentiation of genotypes have been also reported in Thailand (12.8%) (Sakulwira *et al.,* 2003). In Vietnam, CCoV-II, especially CCoV-IIb, may be more predominant among the dog population than CCoV-I. However, CCoV-I infection in Vietnamese dogs should be examined by RT-PCR using further primer sets specific for CCoV-I.

Both CCoV-IIa and IIb cause enteritis and the lesion is limited to the small intestine (Licitra *et al.,* 2014b). On the other hand, pantropic CCoV-IIa spread systemically and caused hemorrhagic gastroenteritis, neurological signs and leukopenia (Decaro *et al.,* 2007; Licitra *et al.,* 2014a). CCoV-IIb also spread

systemically and infected to internal organs, when it co-infected with canine parvovirus (Ntafis *et al.,* 2011; Licitra *et al.,* 2014a). However, there were no significant differences in CCoV infection between diarrheic dogs and healthy dogs. CCoV infection may not be a serious problem in Vietnamese dogs. Further studies will be required to clarify the pathogenicity of CCoV.

Phylogenetic and sequence analysis based on S genes showed that Vietnamese CCoV-IIa (CCoV/dog/HCM27/2014) and CCoV-IIb (CCoV/dog/HCM47/2015) were smilar to CCoVs in China, Chinese ferret badger CoV/DM95/2003 and raccoon dog CoV/GZ43/2003, respectively (Fig.3.2). These Chinese CoVs were found in live animal markets in Shenzhen and Guangzhou, Guangdong Province, China (Vijaykrishna *et al.,* 2007), suggesting that Vietnamese CCoVs might have the same ancestor as these Chinese CCoVs. In our previous study, canine distemper virus in Vietnam was also similar to Chinese viruses (Nguyen *et al.,* 2016).

We succeeded in isolation of CCoV/dog/HCM47/2015 and plague-purified the isolate three times for further characterization. Surprisingly, the plaque-purified CCoV had a large deletion in the ORF3abc and partial E genes (1,165 nt) and a short deletion of ORF7b (60 nt) (Fig. 3). Deletions in ORF3a (47 nt) and ORF3b (31 nt) in CCoV 1-71 (Ma *et al.,* 2008), a deletion in ORF3b (38 nt) in CB/05 (Decaro *et al.,* 2007) and a deletion in ORF7b (154 nt) in 341/05 (Decaro *et al.,* 2010) have also been reported. These data indicate that ORF3abc and ORF7b are not essential and that they are stable in viral replication *in vitro*, but not *in vivo*. In feline coronavirus, it was reported that ORF3abc plays an important role in the efficient macrophage and monocyte tropism (Balint *et al.,* 2012) and that deletion of ORF7b is correlated with a loss of virulence (Herrewegh *et al.,* 1995). In our preliminary data, two dogs orally inoculcated with plaque-purified CCoV/dog/HCM47/2015 did not show any clinical

signs, but anti-CCoV antibody increased after challenge. Now, we are examining the virulence of this plaque-purified CCoV. Importantly, virus isolation is important for characterization, but rapid adaptation by passage of coronaviruses should be monitored.

3.6. Conclusion

In conclusion, this is the first characterization of CCoV in Vietnam. CCoV-IIa and CCoV-IIb are co-circulating among domestic dogs in Vietnam. The plaque-purified CCoV/dog/HCM47/2015 may be a good tool for diagnosis of CCoV infection, because of its rapid viral growth *in vitro* and antigenicity. Furthermore, the virus is a candidate for inactivated and/or attenuated live vaccines, because genetic markers in ORF3abc and 7b are able to differentiate it from field isolates.

3.7. Figure, legends and table

Fig.3.1. Phylogenetic analysis based on 5'-terminal region of spike genes (476 nt).

Vietnamese CCoVs in feces collected from diarrheic and healthy dogs are shown in bold. GeneBank accession numbers of reference strains are as follows: CCoV-II: SK378 (KC175341), K378 (KC175340), Insavc-1 (D13096.1), BGF10 (AY342160.1), NA/09 (JF682842.1), CB/05 (KP981644.1), fc1 (AB781790), TN449 (JQ404410), 171 (KC175339), Chinese ferret CoV/DM95/2003 (EF192156.1), NTU-336 (GQ477367), 430/07 (EU924790), 174/06 (EU856362), 341/05 (EU856361.1), 66/09 (HQ450376), 68/09 (HQ450377.1), 119/08 (EU924791.1) and Raccoon dog CoV/GZ43/2003 (EF192155). FCoV-I: UU20 (HQ392471.1), UU31 (HQ012371.1) and Yayoi (AB695067.1); FCoV-II: NTU156 (GQ152141.1), DF-2 (DQ286398.1), M91-267 (AB781788.1) and Tokyo/cat/130627 (AB907624.1**)**. CCoV-I: 23/03 (KP84972.1) and Elmo/02 (AY307020.1); TGEV**:** Purdue (DQ811789.2) and TS (DQ201447).

Fig.3.2. Phylogenetic analysis based on full length of S gene of CCoV.

Vietnamese isolated and detected CCoV strains are shown in bold. GeneBank accession numbers of reference strains are as follows: CCoV-II: SK378 (KC175341), K378 (KC175340), Insavc-1 (D13096.1), BGF10 (AY342160.1), NA/09 (JF682842.1), CB/05 (KP981644.1), fc1 (AB781790), TN449 (JQ404410), 171 (KC175339), DM95/2003 (EF192156.1), NTU-336 (GQ477367), 430/07 (EU924790), 174/06 (EU856362), 341/05 (EU856361.1), 66/09 (HQ450376), 68/09 (HQ450377.1), 119/08 (EU924791.1) and Raccoon dog CoV/GZ43/2003 (EF192155). FCoV-I: UU20 (HQ392471.1), UU31 (HQ012371.1) and Yayoi (AB695067.1). FCoV-II: NTU156 (GQ152141.1), DF-2 (DQ286398.1), M91-267 (AB781788.1) and

Tokyo/cat/130627 (AB907624.1). CCoV-I: 23/03 (KP84972.1) and Elmo/02 (AY307020.1); TGEV: Purdue (DQ811789.2) and TS (DQ201447).

Fig.3.3. Schema of 3'-terminal one-third of CCoV/dog/HCM47/2015.

Nucleotides sequences of CCoV/dog/HCM47/2015 in fecal sample (A) and plaque-purified virus (B) were analyzed. Plaque-purified CCoV has a large deletion in ORF3abc and partial E genes (1,165 nt), and a small deletion in the ORF7b gene (60 nt) in comparison with the original CCoV in fecal samples.

Table 3.1. Prevalence of anti-CCoV-II antibody among 201 domestic dogs in Ho Chi Minh city, Vietnam

		No. of examined dogs	No. of positive dogs	Ratio of positive dogs (%)
Sex	Female	101	45	44.6
	Male	100	42	42.0
Age	<1	28	8	28.6[a]
	1	44	14	31.8
	2	54	26	48.1
	≥3	75	39	52.0[a]
Breed	Vietnamese	120	47	39.2[b]
	Mixed	51	21	41.2
	Other	30	19	63.3[b]
Total		201	87	43.3

a, b: Significant difference (p<0.05)

Table 3.3. Comparison of VN antibodies against CCoV-IIa fc1 and CCoV-IIb CCoV/dog/HCM47/2015 among 60 domestic dogs in Ho Chi Minh city, Vietnam

		VN test against CCoV/dog/HCM47/2015		Total
		Positive	Negative	
VN test against fc1	Positive	31	0	31 (51.7%)
	Negative	4	25	29 (48.3%)
Total		35 (58.3%)	25 (41.7%)	60 (100.0%)

Table 3.2. Information on Vietnamese CCoV strains

Strains	Date of collection (Year/Month)	Sex	Breed	Age	Clinical sign	Subgenotype	DDBJ accession number
CCoV/dog/HCM26/2014	2014/1	Male	Vietnamese	2 months	Diarrhea	CCoV-IIb	LC190901
CCoV/dog/HCM27/2014	2014/1	Male	Vietnamese	3 months	Diarrhea	CCoV-IIa	LC190906
CCoV/dog/HCM41/2015	2015/6	Female	Mixed	2.5 years	Healthy	CCoV-IIb	LC190902
CCoV/dog/HCM45/2015	2015/6	Male	Mixed	1.5 year	Healthy	CCoV-IIb	LC190903
CCoV/dog/HCM47/2015	2015/6	Female	Mixed	6 years	Healthy	CCoV-IIb	LC190907
CCoV/dog/HCM49/2015	2015/6	Female	Mixed	6 years	Healthy	CCoV-IIb	LC190904
CCoV/dog/HCM60/2015	2015/6	Male	Mixed	4 years	Healthy	CCoV-IIb	LC190905

Fig.3.1

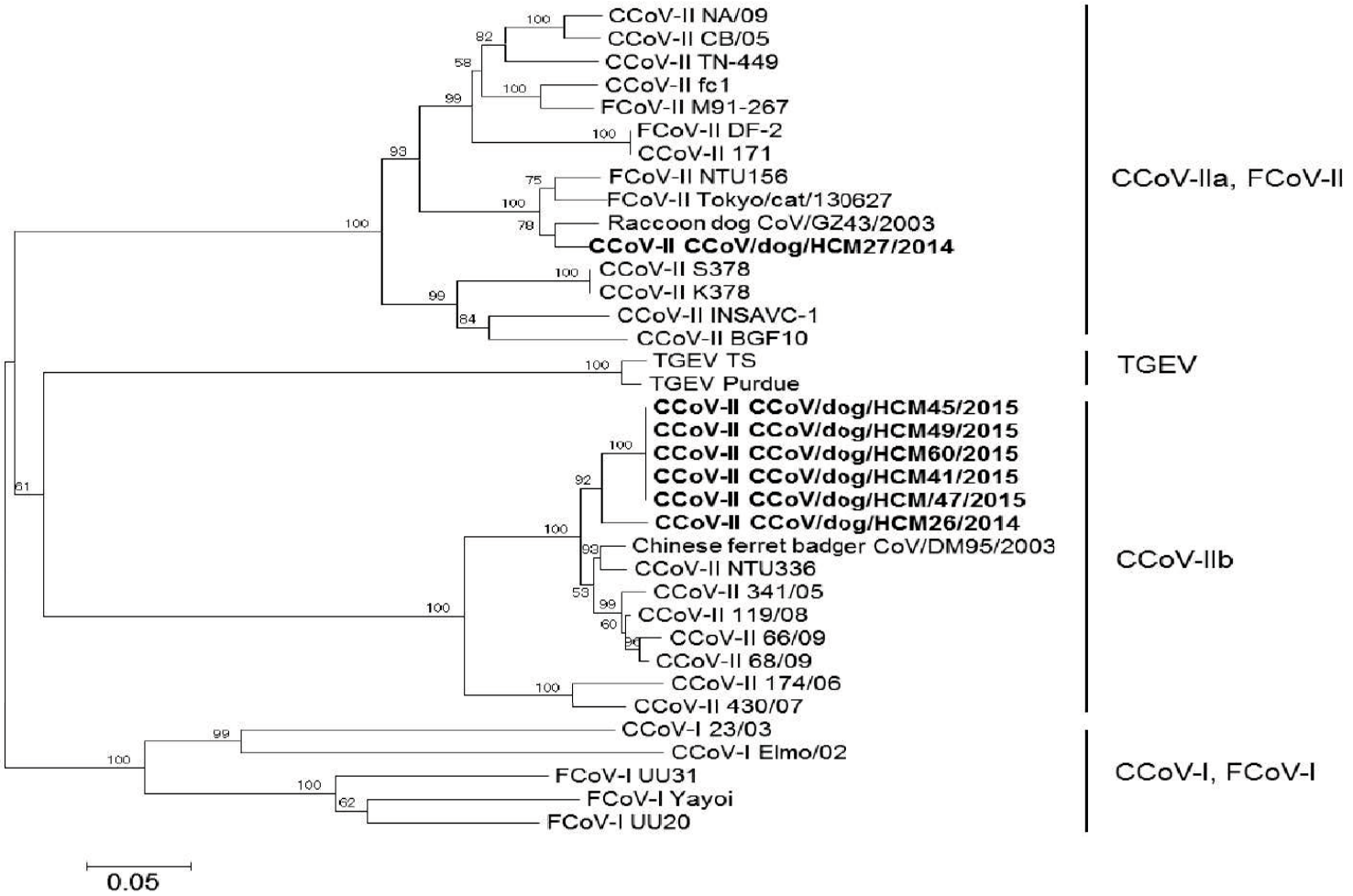

CCoV-II NA/09
CCoV-II CB/05
CCoV-II TN-449
CCoV-II fc1
FCoV-II M91-267
FCoV-II DF-2
CCoV-II 171
FCoV-II NTU156
FCoV-II Tokyo/cat/130627
Raccoon dog CoV/GZ43/2003
CCoV-II CCoV/dog/HCM27/2014
CCoV-II S378
CCoV-II K378
CCoV-II INSAVC-1
CCoV-II BGF10
TGEV TS
TGEV Purdue
CCoV-II CCoV/dog/HCM45/2015
CCoV-II CCoV/dog/HCM49/2015
CCoV-II CCoV/dog/HCM60/2015
CCoV-II CCoV/dog/HCM41/2015
CCoV-II CCoV/dog/HCM/47/2015
CCoV-II CCoV/dog/HCM26/2014
Chinese ferret badger CoV/DM95/2003
CCoV-II NTU336
CCoV-II 341/05
CCoV-II 119/08
CCoV-II 66/09
CCoV-II 68/09
CCoV-II 174/06
CCoV-II 430/07
CCoV-I 23/03
CCoV-I Elmo/02
FCoV-I UU31
FCoV-I Yayoi
FCoV-I UU20
CCoV-IIa, FCoV-II
TGEV
CCoV-IIb
CCoV-I, FCoV-I
0.05

Fig.3.2

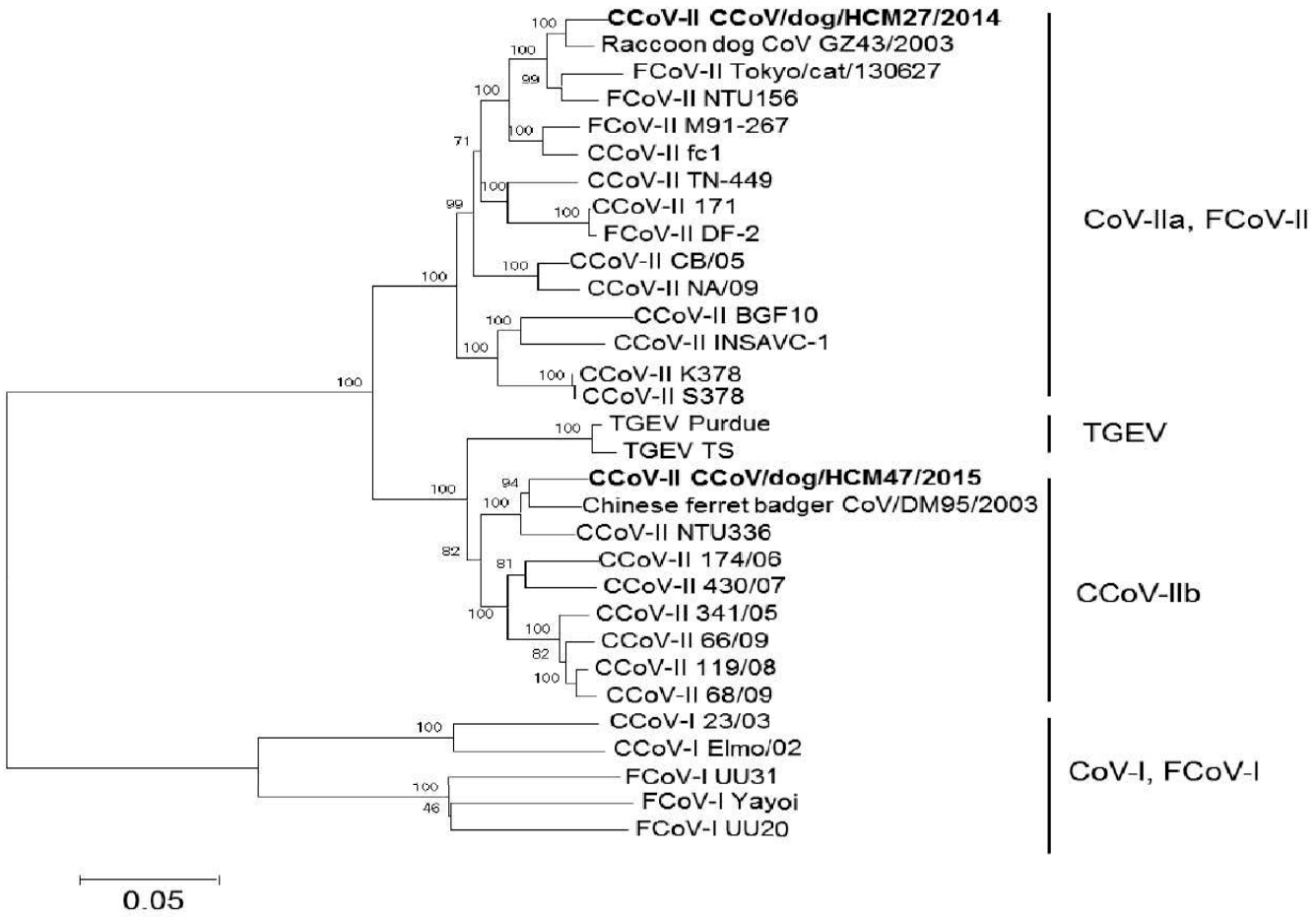
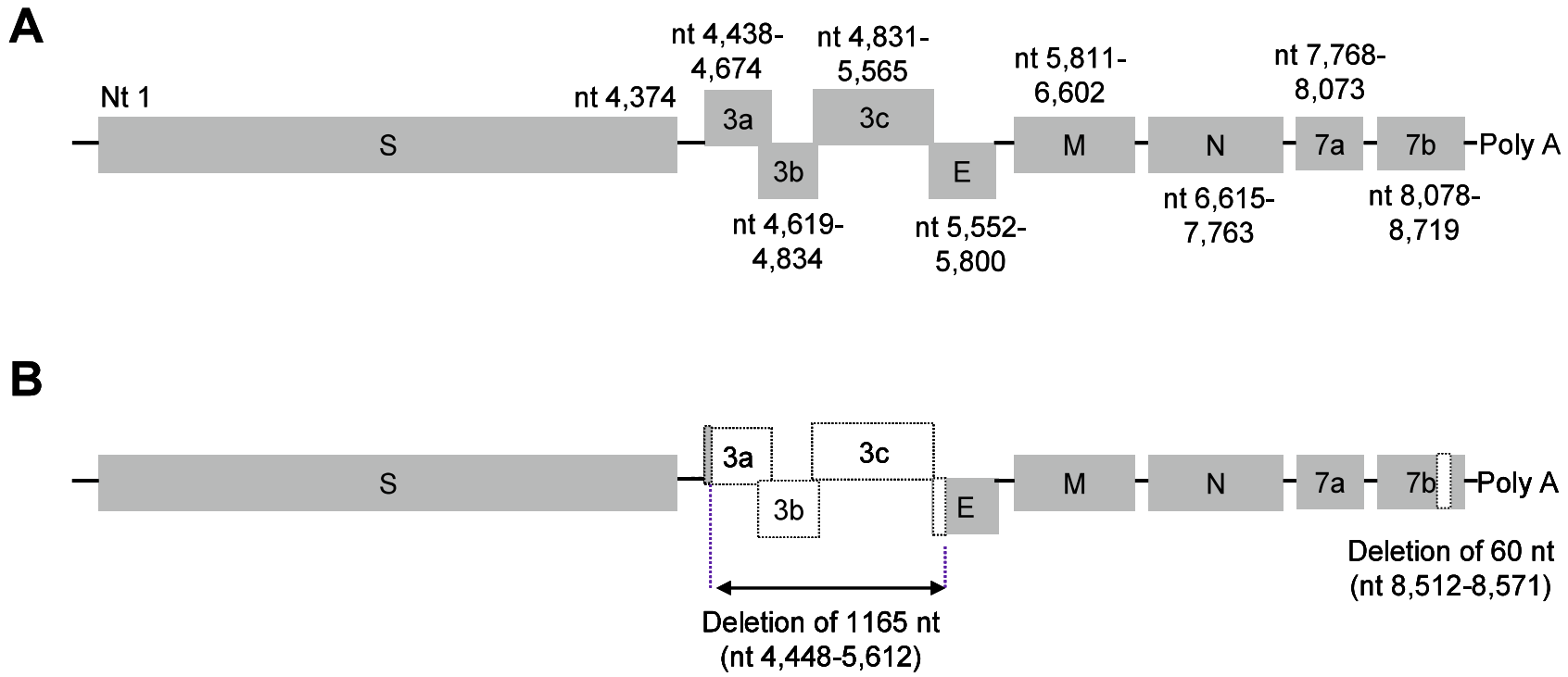

CCoV-II CCoV/dog/HCM27/2014
Raccoon dog CoV GZ43/2003
FCoV-II Tokyo/cat/130627
FCoV-II NTU156
FCoV-II M91-267
CCoV-II fc1
CCoV-II TN-449
CCoV-II 171
FCoV-II DF-2
CCoV-II CB/05
CCoV-II NA/09
CCoV-II BGF10
CCoV-II INSAVC-1
CCoV-II K378
CCoV-II S378
TGEV Purdue
TGEV TS
CCoV-II CCoV/dog/HCM47/2015
Chinese ferret badger CoV/DM95/2003
CCoV-II NTU336
CCoV-II 174/06
CCoV-II 430/07
CCoV-II 341/05
CCoV-II 66/09
CCoV-II 119/08
CCoV-II 68/09
CCoV-I 23/03
CCoV-I Elmo/02
FCoV-I UU31
FCoV-I Yayoi
FCoV-I UU20
0.05
CoV-IIa, FCoV-II
TGEV
CCoV-IIb
CoV-I, FCoV-I

Fig.3.3


A
Nt 1
nt 4,374
nt 4,438-4,674
nt 4,831-5,565
nt 5,811-6,602
nt 7,768-8,073
S
3a
3b
3c
E
M
N
7a
7b
Poly A
nt 4,619-4,834
nt 5,552-5,800
nt 6,615-7,763
nt 8,078-8,719
B
S
3a
3b
3c
E
M
N
7a
7b
Poly A
Deletion of 1165 nt
(nt 4,448-5,612)
Deletion of 60 nt
(nt 8,512-8,571)

4. Chapter 3

Molecular epidemiology of canine parvovirus among domestic dogs in Vietnam

4.1. Abstract

CPV is one of the most important pathogens causing enteritis in domestic dogs. Although there were a few reports of CPV in Vietnam, recent information on CPV infection in domestic dogs in Vietnam is limited. To examine the prevalence of antibody against CPV-2 among Vietnamese dogs, 108 serum samples from dogs without vaccination history were analyzed by virus-neutralization test. The results showed 101 dogs (93.5%) were positive for anti-CPV antibody. To detect genes of CPV, fecal samples collected from 30 diarrheic and 50 healthy dogs were examined by PCR. The prevalence of CPV in diarrheic dogs (43.3%, 13/30) was significantly higher than that in healthy dogs (4.0%, 2/50), indicating that CPV was a major pathogen causing diarrhea in domestic dogs. Genotyping of 15 CPV strains showed that CPV-2a and CPV-2c were circulating in Vietnam and that CPV-2c was a dominant genotype in Vietnam. Virus isolation was performed from fecal samples using A72/cSLAM cells, and nine CPV strains were successfully isolated. In conclusion, the dominant genotypes spreading among Vietnamese dogs transited from CPV2b to CPV-2c.

4.2. Introduction

CPV is one of the most important pathogens of diarrheic disease in dogs. CPV is single-stranded DNA and a member in *Parvoviridae* family*, Protoparvovirus* genus. The genome of CPV encodes three structural proteins (VP1, VP2 and VP3) and two non-structural proteins (NS1 and NS2). VP2 protein can be cleaved of its *N*-amino terminus by host protease to produce VP3 after virion assembly (Agbandje *et al*., 1995; Mirand *et al*., 2016). The VP2 protein plays important roles in the antigenicity and host range of CPVs (Phromnoi S *et al*., 2010).

CPV-2 was first identified in late 1970s as an agent of severe hemorrhagic gastroenteritis in dogs. In 1980s, two antigenic variants were emerged and termed CPV-2a (VP2 87Leu, 101Thr, 300Gly, 305Tyr, 375Asp, 426Asn, 555Ile) and -2b (VP2 87Leu, 101Thr, 300Gly, 305Tyr, 375Asp, 426Asp) (Truyen *et al*., 1996; Decaro *et al*., 2012). CPV-2a and -2b with a mutation at residue 297 from Ser to Ala in VP2 have been named as new CPV-2a and -2b (Martella *et al*., 2005). In 2000, a new genotype, CPV-2c (VP2 426Glu), was recognized in Italy (Buonavoglia *et al*., 2001). Recently, CPV-2c was reported in many countries in Europe, Asia and U.S (Buonavoglia *et al*., 2001; Decaro *et al*., 2007; Hong *et al*., 2007; Decaro *et al*., 2012). In Vietnam, CPV-2b was a predominant genotype in 2004 (Ikeda *et al*., 2000; Nakamura *et al*., 2004). In this study, recent Vietnamese CPV strains circulating in domestic dogs were analyzed.

4.3. Materials and Methods

4.3.1. Serum samples

A total of 108 serum samples from CPV unvaccinated dogs were collected from domestic dogs in Ho Chi Minh city, Vietnam. The information on sex, ages, breed and place of sample collection were recorded. Collected sera were stored at -20^{o}C until analysis.

4.3.2. VN test

To examine seroprevalence of CPV, VN test was performed using feline lymphoblastoid cells (FL-74) as previously described (Ikeda *et al.,* 1998) with modifications. Briefly, VN test was carried out in a 96-wells flat bottom microplate in duplicate. Serum samples were heat-inactivated at 56^{o}C for 30 minutes prior to the assay. Fifty µl of serially diluted serum (1: 100 to 1: 6,400) samples were mixed with $2x10^3$ $TCID_{50}$ /ml CP49 strain (CPV-2, Azetaka *et al.,* 1981) in 50 µl of culture medium. The mixtures were incubated at 37^{o}C with 5% CO_2 for 1 hr. Then, 100 µl of FL74 cells ($5x10^4$ cells/ml) were added to the mixture and incubated at 37^{o}C with 5% CO_2 for 5-7 days. VN titers was defined as the reciprocal of highest serum dilution that protected the cells from showing cytopathic effects (CPE).

4.3.3. Fecal swab samples

A total of 80 fecal swabs were collected from 30 diarrheic and 50 healthy dogs in Ho Chi Minh in Vietnam from 2013 to 2015. The age ranged from 2 months and 13 years old. Swab samples were dissolved in 2 ml of phosphate-buffer saline (PBS), filtrated through the filter of 0.22 µm filter (Millipore, Carrigtwohill, Iceland) and stored at -80^{o}C until analysis.

4.3.4 Viral DNA extraction

Viral DNA were extracted from fecal swab samples using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

4.3.5. Polymerase chain reaction assay (PCR)

For detection of DNA viruses, PCR was carried out using TaKaRa Ex Taq kit (TaKaRa, Otsu, Japan) with the primers 555F (5'- CAGGAAGATATCCAGAAGGA-3') and 555R (5'-GGTGCTAGTTGATATGTAATAAACA-3') (Buonavoglia et al., 2001). PCR condition was follows: first pre-denaturation step at 94ºC for 2 min and follows 40 cycles (at 98ºC for 10 sec, 50ºC for 30 sec, 72ºC for 1 min) and final extension step at 75ºC for 5 min. Amplified products were confirmed as 583bp by electrophoresis on 2% agarose gels, and then purified using QIAquick PCR Purification kit (Qiagen) for sequencing.

4.3.6. Virus isolation

For virus isolation, A72 cells expressing canine SLAM, A72/cSLAM (Nakano *et al*., 2009), were maintained in Dulbecco's minimum essential medium (DMEM; Life Technologies, Carlsbad, CA) containing 10% heat-inactivated fetal calf serum (FBS; Sigma-Aldrich, MO, USA), 100 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies). A72/cSLAM cells in 6 well plates were inoculated with extracts of fecal swabs and then incubated at 37ºC in 5% CO_2 incubator. Cells were observed daily for cytopathic effect (CPE). If there was no CPE, cells were blind-passaged five times. PCR was used for determination of isolated viruses.

4.3.7. Nucleotide Sequencing

To determine the full length of VP2 gene (1,755nt), PCR was performed with primers: VP2FLf (forward), 5'-GTGCAGGACAAGTAAAA-3' (Gallo *et al*., 2012) and 555R (reverse), 5'-GGTGCTAGTTGATATGTAATAAACA-3' (Buonavoglia *et al*., 2001). PCR condition using KOD-Plus Ver.2 kit (TOYOBO, Osaka, Japan) was follows: first pre-denaturation step at 94^{o}C for 2 min, 40 cycles at 98^{o}C for 10 sec, 55^{o}C for 30 sec, 68^{o}C for 2 min and final extension step at 68^{o}C for 5 min. To determine nearly complete genome of isolated CPV (CPV/dog/HCM7/2013 and CPV/dog/HCM22/2013), PCR was performed to amplification of overlapping fragments of with primers sets: 210F (forward), 5'-AGACCGTTACTGACATTCGC-3' and 3469R (reverse), 5'-GTGCCACTAGTTCCAGTATGAG-3'. PCR condition using KOD-Plus Ver.2 kit (TOYOBO) was follows: first pre-denaturation step at 94^{o}C for 2 min, 40 cycles at 98^{o}C for 10 sec, 50^{o}C for 30 sec, 68^{o}C for 4 min and final extension step at 68^{o}C for 10 min. The PCR products were purified using QIAquick PCR Purification kit (Qiagen) for sequencing.

4.3.8. Phylogenetic and statistical analysis

Phylogenetic trees were constructed using the Neighbor-Joining method in MEGA 6.0 (Tamura *et al*., 2013). Bootstrap values were calculated on 1,000 replicates**.** Fisher's exact and Chi-square tests were used for analysis of data. P values of <0.05 were considered to be statistically significant.

4.4. Results

4.4.1. Seroprevalence of CPV infection in Vietnamese dogs

Many dogs (93.5%; 101/108) were positive for antibody against CPV-2. There was no significant difference in seroprevalence of CPV infection among sex, age, breed and area (Table 4.1).

4.4.2. Detection of CPVs in diarrheic and healthy dogs

CPV-2 was detected from 13 diarrheic dogs (43%, 13/30) and two healthy dogs (4%, 2/50). Prevalence of CPV in the diarrheic dogs was significantly higher than that in the healthy dogs ($p<0.05$).

4.4.3. Genotyping of CPV strains in Vietnamese dogs

For determination of CPV genotypes, the nucleotide sequences of partial VP2 gene (519 nt) of 15 CPV-positive samples were analyzed. The results showed that 14 Vietnamese CPV strains possessed Glutamic acid at the 426 position of VP2 protein, indicating that these CPV strains belonged to CPV-2c (Tables 4.2 , 4.3 and 4.4). One CPV strain, CPV/dog/HCM22/2013, belonged to genotype 2 or 2a, when partial VP2 gene was determined (Asparagine at 426 position of VP2 protein). To determine genotype of strain CPV/dog/HCM22/2013 strain, the full length of VP2 gene (1,755 nt) was analyzed. The results showed that CPV/dog/HCM22/2013 strain belonged to new CPV-2a (Tables 4.2 and 4.3).

4.4.4.Virus isolation and their nucleotide sequences

A total of 9 CPV strains were successfully isolated from fecal swab samples. Full length of VP2 gene of seven isolates including CPV/dog/HCM2/2013, CPV/dog/HCM5/2013, CPV/dog/HCM6/2013, CPV/dog/HCM8/2013, CPV/dog/HCM13/2013, CPV/dog/HCM14/2013, CPV/dog/HCM20/2013, and nearly

complete genomes of two representative CPV strains, CPV/dog/HCM7/2013 (CPV-2c) and CPV/dog/HCM22/2013 (new CPV-2a), were determined. In comparison with CPV-2 (CPV-b strain), Vietnamese CPV-2c CPV/dog/HCM7/2013 had two mutations in NS1 protein, I60V (Ile to Val) and L630P (Leu to Pro) and 11 mutations in VP2, A5G (Ala to Gly), M87L (Met to Leu), I101T(Ile to Thr), F267Y (Phe to Tyr), S297A (Ser to Ala), A300G (Ala to Gly), D305V (Asp to Val), Y324I (Tyr to Ile), Q370R (Gln to Arg), N375D (Asn to Asp) and N426E (Asn to Glu), and Vietnamese CPV-2a (CPV/dog/HCM22/2013) had 3 mutations in NS1 protein, including A13G (Ala to Gly), V115I (Val to Ile) and N624K (Asn to Lys) and 9 mutations in VP2, including M87L (Met to Leu), I101T (Ile to Thr), F267Y (Phe to Tyr), S297A (Ser to Ala), A300G (Ala to Gly), D305V (Asp to Val), Y324I (Tyr to Ile), N375D (Asn to Asp) and T440A (Thr to Ala) (Table 4.3).

4.4.5. Phylogenetic analysis

Phylogenetic analysis of VP2 genes showed that Vietnamese CPV-2a was similar to Chinese and Uruguayan new CPV-2a strains and Vietnamese CPVs-2c were similar to Chinese CPV-2c strains (Fig.4.1). In addition, phylogenetic analysis based on nearly complete genome also showed that Vietnamese CPV-2a and CPV-2c were similar to Chinese strains (Data not shown).

4.5. Discussion

To date, CPV infection in dogs with dirrhea have been reported in Nigeria (40.0%) (Chollom *et al*., 2013), Japan (45.9%) (Soma *et al*., 2013), China (58.0%) (Yi *et al*., 2016) and Hungary (84.0%) (Csagola *et al*., 2014). In this study, prevalence of CPV in diarrheic dogs (43.3%) was significantly higher that that in healthy dogs

(4.0%). Serological surveys in Vietnamese dogs also revealed that CPV have been circulated among dog population in Vietnam (93.5%) as well as in Chile (78.0%) (Jamett *et al.,* 2015), India (88.0%) (Belsare *et al.,* 2014), Zimbabwe (84.0%) (McRee *et al.,* 2014), Portugal (71.6%) (Castanheira *et al.,* 2014) and Korea (93.8%) (Yang *et al.,* 2010). These results indicated that CPV was spreading in Vietnam and caused diarrhea to dogs. In addition, seroprevalence in Vietnam was not different between sexes, ages, breeds and areas which is consistent with the recent report that there was no significant difference between sexes or breeds in unvaccinated dogs (Saasa *et al*., 2016).

In Vietnam, CPV-2b was a major genotype in leopards and domestic cats in 2000, (Ikeda *et al*., 2000). In 2004, seven CPV-2b and one CPV-2c were detected (Nakamura *et al*., 2004). CPV-2a and CPV-2b were the predominant genotypes in Asian countries and Australia (Meer *et al*., 2007; Lin *et al*., 2014) and CPV-2c was a minor genotype. Recently, CPV-2c was detected as a predominant genotype in many European countries such as Italy, Germany and Spain (Buonavoglia *et al*., 2001; Decaro *et al*., 2012; Gallo *et al*., 2012; Cságola *et al*., 2014). However, 14 CPV-2c and one CPV-2a were detected in Vietnam in 2013-2015. These data suggested that CPV-2c is a predominant genotype in Vietnam and recently dominant genotype transited from CPV-2b to CPV-2c.

All Vietnamese CPV-2c strains had four amino acid mutations at residue 5 (Ala to Gly), 267 (Phe to Tyr), 324 (Tyr to Ile), 370 (Gln to Arg), and Vietnamese CPV-2a had three amino acid mutations at residue 267 (Phe to Tyr), 324 (Tyr to Ile) and 440 (Thr to Ala) (Table.3). On the other hand, CPVs (genotype 2a and 2b) had a single mutation at residue 5 (Ala to Gly) in cat and 267 (Phe to Tyr) in dog, and 440 (Thr to Ala) in leopard cat (Ikeda *et al*., 2000; Nakamura *et al*., 2004). These data

indicated that there was evolution of CPV in Vietnam. These mutations in Vietnamese CPV-2c strains might affect to antigenicity and pathogenicity of CPV-2c. However, further investigation should be carried out *in vivo.* The mutation at residue 5 (Ala to Gly) was found in cats in Vietnam (Ikeda *et al*., 2000) and in dogs in China (Wang *et al.*, 2016).

In our study, our CPVs had two mutations at residues 324 and 267. Since the residue 323 affects binding to the canine transferin receptor. In addition, the mutation at 267 residue might play an important role in transmission and infection (Chang *et al*., 1992; Xu *et al*., 2012). The mutations at these residue might be lead to change antigenicity and increase spreading widely in domestic dogs in Vietnam. The previous reports showed that virus with mutation at 324 (Tyr to Ile) was detected viral shedding up to 63 days in naturally infected dogs (Lin *et al*., 2014). In addition, all Vietnamese CPV-2c possessed Arginine at 370 position, and this mutation of residue 370 (Gln to Arg) was observed in some Chinese CPV-2a strains (Guo *et al*., 2013) and CPV-2c strains in China (Zhao *et al*., 2015). This residue 370 was close to residues 375 and 377, which are associated with the ability of CPV to hemagglutinate. Residue 370 was also close to residues 379 and 384, which affect binding to viral receptor and host range (Guo *et al*, 2013). Further experiment will be required to clarify the role of this residue 370.

4.6. Conclusion

In this study, high prevalence of CPV was observed in diarrheic dogs in Vietnam, and CPV was confirmed to be a major pathogen to cause enteritis to dogs. In addition, there was transition of dominant genotypes from CPV-2b to CPV-2c with some novel mutations in Vietnam.

4.7. Figures, legends and tables

Fig.1. Phylogenetic tree based on the nucleotide sequences of partial VP2 of CPV. The bootstrap percentages (supported by at least 50% of the 1,000 replicates) are shown above the nodes. The scale bar indicates the number of nucleotide substitutions per site. strains identified in Vietnam are shown in bold. GeneBank accession numbers of reference strains are as follows, CPV 2/2a: CPV-b (M38245.1), CPV-N (M19296.1), CPV-5.us.79 (EU659116.1) and CPV-13.us.81 (EU659118.1). CPV new 2a: V129 (AB054216), V154 (AB054217), UY306 (KM457135.1), UY243 (KM457102.1), UY364 (KM457143.1), CPV/CN/SD9/2014 (KR002802.1), CPV/CN/SD18/2014 (KR002804.1), SC02/2011 (JX660690.1), CPV-LZ1 (JQ268283.1), CPV/CN/JL5/2013 (KR002798.1) and CPV/CN/SD6/2014 (KR002801.1). CPV-2b: 39 (M74849.1), 46 (Z46651.1) and Idaho (U22896.1). CPV new 2b: HCM-18 (AB120722), HNI-2-13 (AB120724), CPV-395 (AY742936.1), CPV-411a.us.98 (EU659120.1), CPV-411b.us.98 (EU659121.1), CPV-410.us.00 (EU659119.1), CPV/CN/HB1/2013 (KR002793.1), CPV/CN/JL3/2013 (KR002796.1), CPV/CN/JL6/2013 (KR002799.1) and CPV-LZ2 (JQ268284.1). CPV-2c: CPV-SD-14-12 (KR611522.1), YANJI-1 (KP749854.1), HNI-4-1 (AB120727), HB (KU508691.1), FH (KU508692.1), LW (KU508693.1), UY370c (KM457142.1), UY72 (KM457107.1), UY173 (KM457115.1) and CPV IZSSI (KU508407.1). Mink enteritis virus: MEV-L (KT899746.1) and Abashiri (D00765.1). Feline panleukopenia virus: FVP-3.us 67 (EU659111.1) and FPV-kai.us.06 (EU659115.1).

Table 4.1. Prevalence of antibody against CPV-2 among domestic dogs in Ho Chi Minh city, Vietnam

		No. of examined dogs	No. of positive dogs	Ratio of positive dogs (%)
Sex	Female	56	51	91.1
	Male	52	50	96.2
Age (year old)	<1	21	21	100.0
	1- < 2	22	19	86.4
	2- < 3	22	21	95.5
	≥3	43	40	93.0
Breed	Vietnamese	68	66	97.1
	Mixed	32	28	87.5
	Other	8	7	87.5
Area	Urban	68	61	89.7
	Suburban	40	40	100
Total		108	101	93.5

Table 4.2. Genotyping of canine parvoviruses based on difference of amino acid in VP2 protein gene

	Amino acid variations in the VP2 protein of canine parvoviruses							
Position	87	101	297	300	305	375	426	555
CPV-2	Met	Ile	Ser	Ala	Asp	Asn	Asn	Val
CPV-2a	**Leu**	**Thr**	Ser	**Gly**	**Tyr**	**Asp**	Asn	**Ile**
CPV-2b	**Leu**	**Thr**	Ser	**Gly**	**Tyr**	**Asp**	**Asp**	Val
New CPV-2a	**Leu**	**Thr**	**Ala**	**Gly**	**Tyr**	**Asp**	Asn	Val
New CPV-2b	**Leu**	**Thr**	**Ala**	**Gly**	**Tyr**	**Asp**	**Asp**	Val
CPV-2c	**Leu**	**Thr**	**Ala**	**Gly**	**Tyr**	**Asp**	**Glu**	Val
Isolated CPV in this study								
CPV/dog/HCM2/2013 (CPV-2c)	**Leu**	**Thr**	**Ala**	**Gly**	**Tyr**	**Asp**	**Glu**	Val
CPV/dog/HCM5/2013 (CPV-2c)	**Leu**	**Thr**	**Ala**	**Gly**	**Tyr**	**Asp**	**Glu**	Val
CPV/dog/HCM6/2013 (CPV-2c)	**Leu**	**Thr**	**Ala**	**Gly**	**Tyr**	**Asp**	**Glu**	Val
CPV/dog/HCM7/2013 (CPV-2c)	**Leu**	**Thr**	**Ala**	**Gly**	**Tyr**	**Asp**	**Glu**	Val
CPV/dog/HCM8/2013 (CPV-2c)	**Leu**	**Thr**	**Ala**	**Gly**	**Tyr**	**Asp**	**Glu**	Val
CPV/dog/HCM13/2013 (CPV-2c)	**Leu**	**Thr**	**Ala**	**Gly**	**Tyr**	**Asp**	**Glu**	Val
CPV/dog/HCM14/2013 (CPV-2c)	**Leu**	**Thr**	**Ala**	**Gly**	**Tyr**	**Asp**	**Glu**	Val
CPV/dog/HCM20/2013 (CPV-2c)	**Leu**	**Thr**	**Ala**	**Gly**	**Tyr**	**Asp**	**Glu**	Val
CPV/dog/HCM22/2013 (New CPV-2a)	**Leu**	**Thr**	**Ala**	**Gly**	**Tyr**	**Asp**	Asn	Val

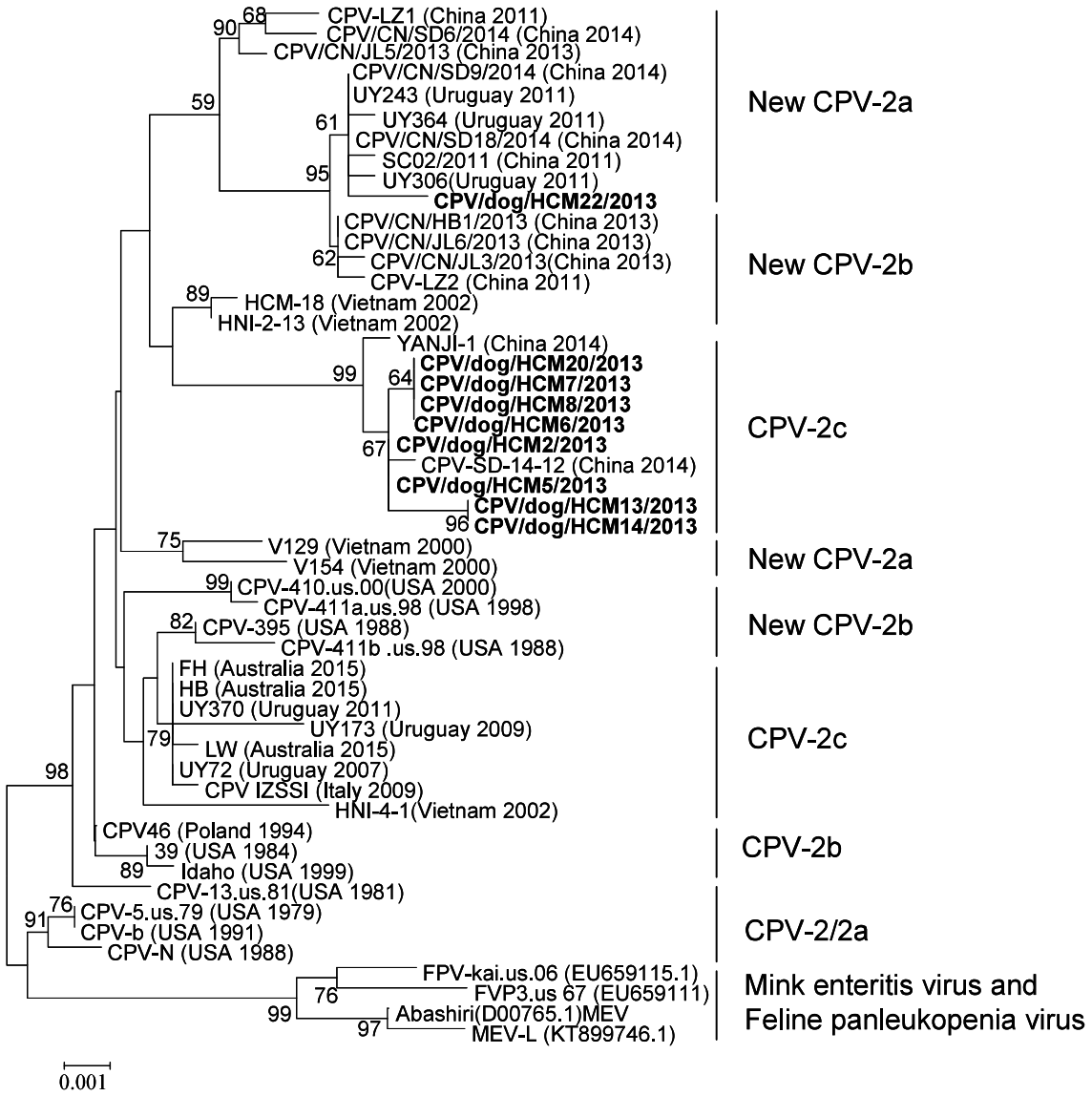
Table 4.3. Variation of amino acids in VP2 among Vietnamese CPVs.

		Mutation of amino acid in VP2 of Vietnamese CPVs													
	Genotype	5	13	87	101	265	267	297	300	305	324	370	375	426	440
CPV-b (M38245)	CPV-2	A	P	M	I	T	F	S	A	D	Y	Q	N	N	T
In previous study in Vietnam															
HNI-2-13 (AB120724)	New CPV-2b	.	.	L	T	.	Y	A	G	Y	.	.	D	D	.
HNI-3-4 (AB120725)	New CPV-2b	.	.	L	T	.	.	A	G	Y	.	.	D	D	.
HNI-3-1 (AB120726)	New CPV-2b	.	.	L	T	.	.	A	G	Y	.	.	D	D	.
HCM-6 (AB120720)	New CPV-2b	.	.	L	T	.	.	A	G	Y	.	.	D	D	.
HCM-8 (AB120721)	New CPV-2b	.	S	L	T	K	.	A	G	Y	.	.	D	D	.
HCM-18 (AB120722)	New CPV-2b	.	.	L	T	.	Y	A	G	Y	.	.	D	D	.
HCM-23 (AB120723)	New CPV-2b	.	.	L	T	.	.	A	G	Y	.	.	D	D	.
HCM-4-1(AB120727)	CPV-2c	.	.	L	T	.	.	A	G	Y	.	.	D	E	.
In this study															
Full length VP2 (Isolated CPV)															
CPV/dog/HCM2/2013	CPV-2c	G	.	L	T	.	Y	A	G	Y	I	R	D	E	.
CPV/dog/HCM5/2013	CPV-2c	G	.	L	T	.	Y	A	G	Y	I	R	D	E	.
CPV/dog/HCM6/2013	CPV-2c	G	.	L	T	.	Y	A	G	Y	I	R	D	E	.
CPV/dog/HCM7/2013	CPV-2c	G	.	L	T	.	Y	A	G	Y	I	R	D	E	.
CPV/dog/HCM8/2013	CPV-2c	G	.	L	T	.	Y	A	G	Y	I	R	D	E	.
CPV/dog/HCM13/2013	CPV-2c	G	.	L	T	.	Y	A	G	Y	I	R	D	E	.
CPV/dog/HCM14/2013	CPV-2c	G	.	L	T	.	Y	A	G	Y	I	R	D	E	.
CPV/dog/HCM20/2013	CPV-2c	G	.	L	T	.	Y	A	G	Y	I	R	D	E	.
CPV/dog/HCM22/2013	New CPV-2a	.	.	L	T	.	Y	A	G	Y	I	.	D	.	A
Partial -length VP2 (Detected CPV)															
CPV/dog/HCM1/2013	CPV-2c	-	-	-	-	-	-	-	-	-	-	-	-	E	.
CPV/dog/HCM4/2013	CPV-2c	-	-	-	-	-	-	-	-	-	-	-	-	E	.
CPV/dog/HCM9/2013	CPV-2c	-	-	-	-	-	-	-	-	-	-	-	-	E	.
CPV/dog/HCM18/2013	CPV-2c	-	-	-	-	-	-	-	-	-	-	-	-	E	.
CPV/dog/HCM82/2015	CPV-2c	-	-	-	-	-	-	-	-	-	-	-	-	E	.
CPV/dog/HCM88/2015	CPV-2c	-	-	-	-	-	-	-	-	-	-	-	-	E	.

Table 4.4. Epidemiological information on CPV infection of domestic dogs in Ho Chi Minh city, Vietnam

Dog. No	Sex	Age	Clinical sign	Collection date (month/year)	Breed	PCR detection	Virus isolation
CPV/dog/HCM1/2013	Male	8 months	Diarrhea, cough, nasal discharge	10/2013	Vietnamese	2c	
CPV/dog/HCM2/2013	Female	12 months	Diarrhea, ocular discharge	10/2013	Japanese	2c	CPV-2c
CPV/dog/HCM4/2013	Female	3 months	Diarrhea, cough	10/2013	Vietnamese	2c	
CPV/dog/HCM5/2013	Male	4 months	Diarrhea	10/2013	Japanese	2c	CPV-2c
CPV/dog/HCM6/2013	Male	6 months	Diarrhea, ocular discharge	10/2013	Vietnamese	2c	CPV-2c
CPV/dog/HCM7/2013	Female	4 months	Diarrhea	10/2013	Mixed	2c	CPV-2c
CPV/dog/HCM8/2013	Male	2 months	Diarrhea, cough	10/2013	Vietnamese	2c	CPV-2c
CPV/dog/HCM9/2013	Female	2 months	Diarrhea, nasal discharge	10/2013	Vietnamese	2c	
CPV/dog/HCM13/2013	Female	3 months	Diarrhea, cough, nasal discharge	11/2013	Vietnamese	2c	CPV-2c
CPV/dog/HCM14/2013	Male	3 months	Diarrhea, cough	11/2013	Vietnamese	2c	CPV-2c
CPV/dog/HCM18/2013	Female	8 months	Diarrhea, cough	11/2013	Japanese	2c	
CPV/dog/HCM20/2013	Male	4 months	Diarrhea	11/2013	Japanese	2c	CPV-2c
CPV/dog/HCM22/2013	Female	2 months	Diarrhea	12/2013	Vietnamese	2a	New CPV-2a
CPV/dog/HCM82/2015	Male	3years old	Healthy	6/2015	Berger	2c	
CPV/dog/HCM88/2015	Male	2years old	Healthy	6/2015	Vietnamese	2c	

Fig.4.1


CPV-LZ1 (China 2011)
CPV/CN/SD6/2014 (China 2014)
CPV/CN/JL5/2013 (China 2013)
CPV/CN/SD9/2014 (China 2014)
UY243 (Uruguay 2011)
UY364 (Uruguay 2011)
CPV/CN/SD18/2014 (China 2014)
SC02/2011 (China 2011)
UY306(Uruguay 2011)
CPV/dog/HCM22/2013
CPV/CN/HB1/2013 (China 2013)
CPV/CN/JL6/2013 (China 2013)
CPV/CN/JL3/2013(China 2013)
CPV-LZ2 (China 2011)
HCM-18 (Vietnam 2002)
HNI-2-13 (Vietnam 2002)
YANJI-1 (China 2014)
CPV/dog/HCM20/2013
CPV/dog/HCM7/2013
CPV/dog/HCM8/2013
CPV/dog/HCM6/2013
CPV/dog/HCM2/2013
CPV-SD-14-12 (China 2014)
CPV/dog/HCM5/2013
CPV/dog/HCM13/2013
CPV/dog/HCM14/2013
V129 (Vietnam 2000)
V154 (Vietnam 2000)
CPV-410.us.00(USA 2000)
CPV-411a.us.98 (USA 1998)
CPV-395 (USA 1988)
CPV-411b .us.98 (USA 1988)
FH (Australia 2015)
HB (Australia 2015)
UY370 (Uruguay 2011)
UY173 (Uruguay 2009)
LW (Australia 2015)
UY72 (Uruguay 2007)
CPV IZSSI (Italy 2009)
HNI-4-1(Vietnam 2002)
CPV46 (Poland 1994)
39 (USA 1984)
Idaho (USA 1999)
CPV-13.us.81(USA 1981)
CPV-5.us.79 (USA 1979)
CPV-b (USA 1991)
CPV-N (USA 1988)
FPV-kai.us.06 (EU659115.1)
FVP3.us 67 (EU659111)
Abashiri(D00765.1)MEV
MEV-L (KT899746.1)
New CPV-2a
New CPV-2b
CPV-2c
New CPV-2a
New CPV-2b
CPV-2c
CPV-2b
CPV-2/2a
Mink enteritis virus and
Feline panleukopenia virus
0.001

5. Chapter 4

Molecular epidemiology of enteric viruses among domestic dogs in Vietnam

5.1. Abstract

Vietnamese MRVs and CKoVs were characterized. The prevalence of MRV in diarrheic dogs (50%, 15/30) was significantly higher than that in healthy dogs (4%, 2/50). Mix-infection with MRV and the other enteric viruses (CDV, CPV, CKoV) were commonly observed in diarrheic dogs. Four MRVs were isolated from feces, and genetic analysis of MRV S1 genes showed that three Vietnamese MRVs belonged to serotype 3 and one belonged to serotype 2. Vietnamese serotypes 3 and 2 showed the highest identities with Chinese porcine MRV-3 (95.2%-96.1%) and Chinese human MRV-2 (94.6%), respectively. CKoV was detected from three diarrheic (10%) and two healthy dogs (4%). Nucleotide sequence of CKoV showed that Vietnamese CKoV was similar to those in Korea. This is the first report on MRV and CKoV infections in Vietnamese dogs.

5.2. Introduction

Mammalian orthoreovirus (MRV) is a double-stranded (ds) RNA virus belonging to *Orthoreovirus* genus, *Reoviridae* family. MRVs cause symptomatic or asymptomatic infections in mammals and possess broad host range (Decaro *et al.,* 2005). Currently, MRVs were divided into four serotypes, including serotype 1 (Lang), serotype 2 (D5/Jones), serotype 3 (Dearing) and serotype 4 (Ndelle), by VN and HI tests (Sabin *et al.,* 1959; Knipe *et al.,* 2013). MRV contains 10 genome segments, which are named as large (L1, L2 and L3), medium (M1, M2 and M3) and small (S1, S2, S3 and S4) based on their electrophoresis mobility (Nibert *et al.,* 1990). The S1 gene encodes the σ1 protein which located on the outer capsid of the virion and is responsible for viral attachment to cellular receptors and determination of serotypes (Decaro *et al.,* 2005). MRVs have been isolated from domestic dogs in U.S., Japan and Italy (Lou *et al.,* 1963, Murakami *et al.,* 1979; Kobubu et al, 1993, Decaro *et al.,* 2005).

Canine kobuvirus (CKoV), a single-stranded positive RNA virus belonging to *Kobuvirus* genus, *Picornaviridae* family. *Kobuvirus* genus is divided into three species, Aichivirus A (Yamashita *et al.,* 1991), Aichivirus B (formerly Bovine kobuvirus) and Aichivirus C (formerly Porcine kobuvirus). CKoV may be considered to be a genotype CaKV type 1 different from murine kobuvirus MuKV type 1 and human Aichivirus type 1 (Martino *et al.,* 2013). It is reported that CKoV was associated with diarrehea in domestic dogs (Li *et al.,* 2016)**.**

In Vietnam, no information on MRV and CKoV infection are available in domestic dogs. In this study, MRV and CKoV in domestic dogs were analyzed.

5.3. Materials and Methods

5.3.1. Fecal swab samples

A total of 80 fecal swabs were collected from 30 diarrheic and 50 healthy dogs in animal hospital and households, respectively, in Ho Chi Minh, Vietnam from 2013 to 2015. The ages were between 2 months and 13 years old. The swab samples were dissolved in 2 ml phosphate-buffered saline (PBS), filtrated through a 0.22 μm filter (Millipore, Carigtwohill, Iceland) and stored at -80^{o}C until analysis.

5.3.2. Viral RNA extraction

Viral RNA were extracted from fecal swab samples with QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

5.3.3. Reverse transcription polymerase chain reaction (RT-PCR)

For detection of RNA viruses, RT-PCR were performed by using QIAGEN One step RT-PCR (Qiagen) with primers for MRV, L1-rv5, 5'- GCA TCC ATT GTA AAT GAC GAG TCT G-3' and L1-rv6, 5'- CTT GAG ATT AGC TCT AGC ATC TTC TG-3', and primers for CKoV, CKoVF, 5'-CTC CCC TCA GCT GCC TTC TC-3' and CaKoVR, 5'-GAG GAT CTG AAA TTT GGA AG-3' (Leary *et al.,* 2002; Choi *et al.,* 2014). One-step RT-PCR was carried out in 40 sequential cycles at 94^{o}C for 30 sec, 50^{o}C (for MRV) or 52^{o}C (for CKoV) for 30 sec and 72^{o} C for 1 min after incubation step for the synthesis of cDNA at 50^{o}C for 30 min and then at 95^{o}C for 15 min. The amplified products were comfirmed as 416 bp for MRV and 252 bp for CKoV by electrophoresis on 2% agarose gels, and then purified using QIAquick PCR Purification kit (Qiagen) for sequencing.

5.3.4. Virus isolation

A72 cells expressing canine SLAM, A72/cSLAM (Nakano *et al*., 2009) were maintained in Dulbecco's minimum essential medium (DMEM; Life Technologies, Carlsbad, CA, USA) containing 10% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich, MO, USA), 100 units/ml penicillin and 100 µg/ml streptomycin (Life Technologies). A72/cSLAM cells in 6 well plate were inoculated with extracts from fecal swab samples and then incubated at 37^{o}C in 5% CO_2 incubator. Cells were daily observed for cytopathic effect (CPE). If there was no CPE, the cells were blind-passaged five times. RT-PCR were used for determination of isolated enteric viruses.

5.3.5. Genotyping of isolated mammalian orthoreovirus

To determine genotype of MRV, RT-PCR was carried out using primers S1F, 5'-GCT ATT SGY RCB KAT G-3' and S1R, 5'- GAT KRR WNB CCV YHG TGC CG-3', for amplification of S1 gene of MRV-1 and MRV-2. Primers S1-R3F, 5'-TGG GAC AAC TTG AGA CAG GA-3' and S1R were used for amplification of S1 of MRV-3. One step RT-PCR was carried out in 40 sequential cycles at 94^{o}C for 30 sec, 53^{o}C for 30 sec and 72^{o}C for 2 min after incubation step for the synthesis of cDNA at 50^{o}C for 30 min and at 95^{o}C for 15 min.

5.3.6. Phylogenetic and statistical analysis

Phylogenetic trees were constructed using the Neighbor-Joining method in MEGA 6.0 (Tamura K *et al.,* 2013). Bootstrap values were calculated on 1,000 replicates. Fisher's exact and Chi-square tests were used for analysis of data. *P* values of <0.05 were considered to be statistically significant.

5.4. Results

5.4.1. Detection of MRV and CKoV from diarrheic and healthy dogs

Prevalence of MRV in diarrheic dogs (50%, 15/30) was significantly higher than that in healthy dogs (4%, 2/50). There was no difference of prevalence of CKoV between diarrheic (10%, 3/30) and healthy dogs (4%, 2/50) (Table 5.2). Single MRV infection was observed in 3 diarrheic dogs, double infection with MRV and CPV or CDV was in 8 dogs, triple infection with MRV, CPV and CDV was in 2 dogs, triple infection with MRV, CDV and CKoV was in one dog and quadruple infection of MRV, CDV, CPV and CKoV was in one dog (Table 5.1).

5.4.2. Virus isolation and nucleotide sequence analysis of Vietnamese MRVs

For isolation of MRV, four MRVs were successfully isolated and named as MRV/dog/HCM9/2013, MRV/dog/HCM16/2013, MRV/dog/HCM19/2013 and MRV/dog/HCM69/2013. In A72/cSLAM cells, MRV caused CPE on 3-4 days post-inoculation. MRV-infected cells showed granulating, shrinking, rounding and detachment (Fig.5.3). Phylogenetic analysis using S1 genes indicated that one Vietnamese MRV strain, MRV/dog/HCM9/2013, belonged to serotype 2, and the other three MRVs belonged to serotype 3. Vietnamese MRV-2 was similar to human MRV-2 (302II strain) in China (94.6% identity in amino acid), and Vietnamese MRV-3 were to porcine MRV-3 (SC-A, GD-1 strains) in China (95.2%-96.1% identity in amino acid) (Table 5.3, Fig.5.1). The identities of the nucleotide and amino acid sequences of S1 genes among three Vietnamese MRVs-3 ranged from 98.0%-99.5%, and 97.3%-98.6%, respectively.

5.4.3. Nucleotide sequence analysis of Vietnamese CKoV

Unfortunately, Vietnamese CKoV could not be isolated. Five CKoV strains were detected from fecal samples and named as CKoV/dog/HCM9/2013, CKoV/dog/HCM13/2013, CKoV/dog/HCM16/2013, CKoV/dog/HCM47/2015 and CKoV/dog/HCM69/2015. Nucleotide sequences of partial 3D gene of five CKoVs were determined. Phylogenetic analysis of CKoV based on partial 3D genes showed that Vietnamese CKoVs belonged to canine group and was similar to CKoV in Korea (CKoV-K73, CKoV-K-71, CKoV-K-78, 12D049) and UK (UK003) (Fig.5.2). Identities among five Vietnamese CKoVs were ranged from 97.2-100%, those between Vietnamese CKoVs and Korean CKoVs were 95.8%-99.5% and those between Vietnamese CKoVs and UK CKoVs were 95.8%-96.2%.

5.5. Discussion

Prevalence of MRV infection in diarrheic dogs (50%, 15/30) was significantly higher than that in healthy dogs (4%), but co-infection with other enteric viruses was observed in many diarrheic dogs (Tables 5.1 and 5.2). Dogs experimentally infected with MRV-1 strain, IU41, which was isolated from urine of healthy adult dog in 1970 in Japan, did not show any clinical sign (Murakami *et al.,* 1979). On the other hand, dogs experimentally infected with MRV-1 isolated from the dead dog with choking, bubbling of foamy saliva, vomiting and bloody diarrhea developed clinical signs, nasal discharge, foamy saliva, cough and fever (39.5^{o}C-40^{o}C) (Lou TY *et al.,* 1963). These results indicated that pathogenicity of MRV has been still unclear, but MRV might be one of exacerbation factors of diarrhea.

In Vietnam, 10% of diarrheic dogs and 4% of healthy dogs were infected with CKoV. There was no significant difference between diarrheic dogs and healthy dogs. Similar results were reported in Korea or Japan (Oem *et al.,* 2014; Soma *et al.,* 2016).

In Vietnam, mix-infection of CKoV and MRV or CPV were observed, while mix-infection of CKoV and CDV or CCoV were commonly recognized in Korea (Oem *et al.,* 2014). These results indicated that CKoV might be a minor pathogen of enteritis in dogs.

5.6. Conclusion

We succeeded in detection of Vietnamese MRVs and CKoVs and isolation of MRVs.

5.7. Figure legends and tables

Fig.5.1. Phylogenetic analysis based on partial S1 genes of MRV.

The bootstrap percentages (supported by at least 50% of the 1,000 replicates) are shown above the nodes. The scale bar indicates the number of nucleotide substitutions per site. Vietnamese isolated MRVs are shown in bold and red. Genbank accession number of references strains are as follows. MRV-1: Lang (M35963.1), HB-A (KC462155.1), HB-C (KF013857.1), T1/T28/KM/2013 (KP185123.1), SHR-A (JX415469.1) and MRV00304/2014 (KJ676385.1). MRV-2: Jones (M35964.1), BYL (EU049606.1), BYD1 (DQ312301.1), JP (EU049607.1), 729 (JN799419.1), T2W ((DQ220017.1), RpMRV-YN2012 (KM087111.1), WIV3 (KT444578.1), 302I cell (EU049603.1) and 302II (EU049604.1). MRV-3: Dearing (M10262.1), KPR150 (F829217.1), KPR155 (JF829218.1), KPR A (JF829220.1), SC-A (DQ911244.1), GD-1(JX486063.1), SD-14 (KT224510.1), T3/T28/KM/2013 (KP185124.1), STS3 (M32862.1), T3 (JQ979273.1), T3D/04 (AY785910.1), SI-MRV01 (KF154730.1) and T3/Bat/Germany/342/08 (JQ412761.1). MRV-4: (AF368035.1).

Fig.5.2. Phylogenetic tree based on the partial RdRp genes of CKoV.

The bootstrap percentages (supported by at least 50% of the 1,000 replicates) are shown above the nodes. The scale bar indicates the number of nucleotide substitutions per site. CKoV identified in Vietnam are shown in bold and red. GeneBank accession numbers of reference strains are as follows, CKoV-71 (KF663763), CKoV-73(KF663765), CKoV-78(KF663766), 12D049 (KF924623), CKoV-K7 (KF663755), UK003 (KC161964), C.DD2 (KM068048), CKoV-009 (JN394542), CKoV-083 (JN394543), AN211 (JN387133), 12Q87-2 (KC894952), M-5 (JF755427), M166/91(AB097165), SH-W-CHN(JN630514), KBH71(HQ650187) and TM003k (HM228882).

Fig.5.3. Cytopathic effect (CPE) of Vietnamese MRV in cell culture.

A) Uninfected cells control. B) CPE observed in A72/cSLAM cells infected with MRV/dog/HCM9/2013 strain.

Table 5.1. Epidemiological information of positive dogs for MRV and CKoV.

ID	Sex	Age (month)	Clinical sign	Collection date (month/year)	Breed	RT-PCR detection		Virus isolation	Mix-infection with other enteric viruses*
						MRV	CKoV	MRV	
3	Female	2	Diarrhea, ocular discharge	10/2013	Vietnamese	+	-		
5	Male	4	Diarrhea	10/2013	Japanese	+	-		CPV
6	Male	6	Diarrhea, ocular discharge	10/2013	Vietnamese	+	-		CPV
7	Female	4	Diarrhea	10/2013	Mixed	+	-		CPV
9	Female	2	Diarrhea, nasal discharge	10/2013	Vietnamese	+	+	MRV-2	CPV, CDV
10	Male	8	Diarrhea	10/2013	Japanese	+	-		CDV
11	Male	3	Diarrhea, cough	11/2013	Japanese	+	-		CDV
12	Male	8	Diarrhea, ocular discharge	11/2013	Vietnamese	+	-		CDV
13	Female	3	Diarrhea, cough, nasal discharge	11/2013	Vietnamese	-	+		CPV
14	Male	3	Diarrhea, cough	11/2013	Vietnamese	+	-		CDV, CPV
15	Female	2	Diarrhea	11/2013	Japanese	+	-		
16	Male	4	Diarrhea	11/2013	Mixed	+	+	MRV-3	CDV
17	Female	4	Diarrhea	11/2013	Japanese	+	-		
18	Female	8	Diarrhea, cough	11/2013	Japanese	+	-		CPV
19	Male	3	Diarrhea, nasal discharge	11/2013	Vietnamese	+	-	MRV-3	CDV
20	Male	4	Diarrhea	11/2013	Japanese	+	-		CPV, CDV
47	Female	72	Healthy	6/2015	Mixed	-	+		CCoV
52	Female	6	Healthy	6/2015	Vietnamese	+	-		CDV
69	Male	24	Healthy	6/2015	Vietnamese	+	+	MRV-3	
Total						17	5		

CPV, canine parvovirus; CDV, canine distemper virus; CCoV, canine coronavirus; MRV, mammalian orthoreovirus; CKoV, canine bovuvirus; +, positive; -, negative.

*; CPV, CDV and CCoV were detected in the previous studies by PCR or RT-PCR.

Table 5.2. MRV and CKoV infection of diarrheic and healthy dogs by RT-PCR

		RT-PCR	
		MRV	CKoV
Diarrheic dogs	No. of examined dogs	30	30
	No. of positive dogs	15	3
	Ratio of positive dogs (%)	50.0[a]	10.0
Healthy dogs	No. of examined dogs	50	50
	No. of positive dogs	2	2
	Ratio of positive dogs (%)	4.0[a]	4.0

a: Significant different *(P<0.05)*

Table 5.3. Identities of S1 genes between Vietnamese MRVs and other MRV strains (nt/aa)

Strain	MRV propotype strains				Bat reovirus		Human reovirus		Pig reovirus	
	T1L	T2J	T3D	T4N	RpMRV -YN2012	T3/Bat/ 342/08	302II	SI-MRV01	GD-1	SC-A
MRV/dog/HCM9/ 2013	58.5/52.4	62.4/61.8	41.9/25.4	42.6/23.2	41.4/24.5	83.3/91.0	93.2/94.6	41.2/24.3	42.2/24.5	41.4/24.9
MRV/dog/HCM16/ 2013	41.4/27.5	42.8/27.6	84.4/90.6	68.2/66.4	41.6/25.6	79.5/86.9	41.8/24.5	79.9/87.2	93.1/94.3	94.8/95.2
MRV/dog/HCM19/ 2013	41.3/25.6	42.7/27.0	84.2/90.2	68.3/66.8	41.7/25.6	79.4/86.0	41.9/24.9	79.8/87.4	92.9/94.0	94.7/95.2
MRV/dog/HCM69/ 2015	41.0/25.4	42.8/26.8	84.1/90.2	68.3/67.0	42.0/25.4	79.9/87.4	41.8/24.9	80.2/87.9	92.9/94.5	95.0/96.1

Fig.5.1

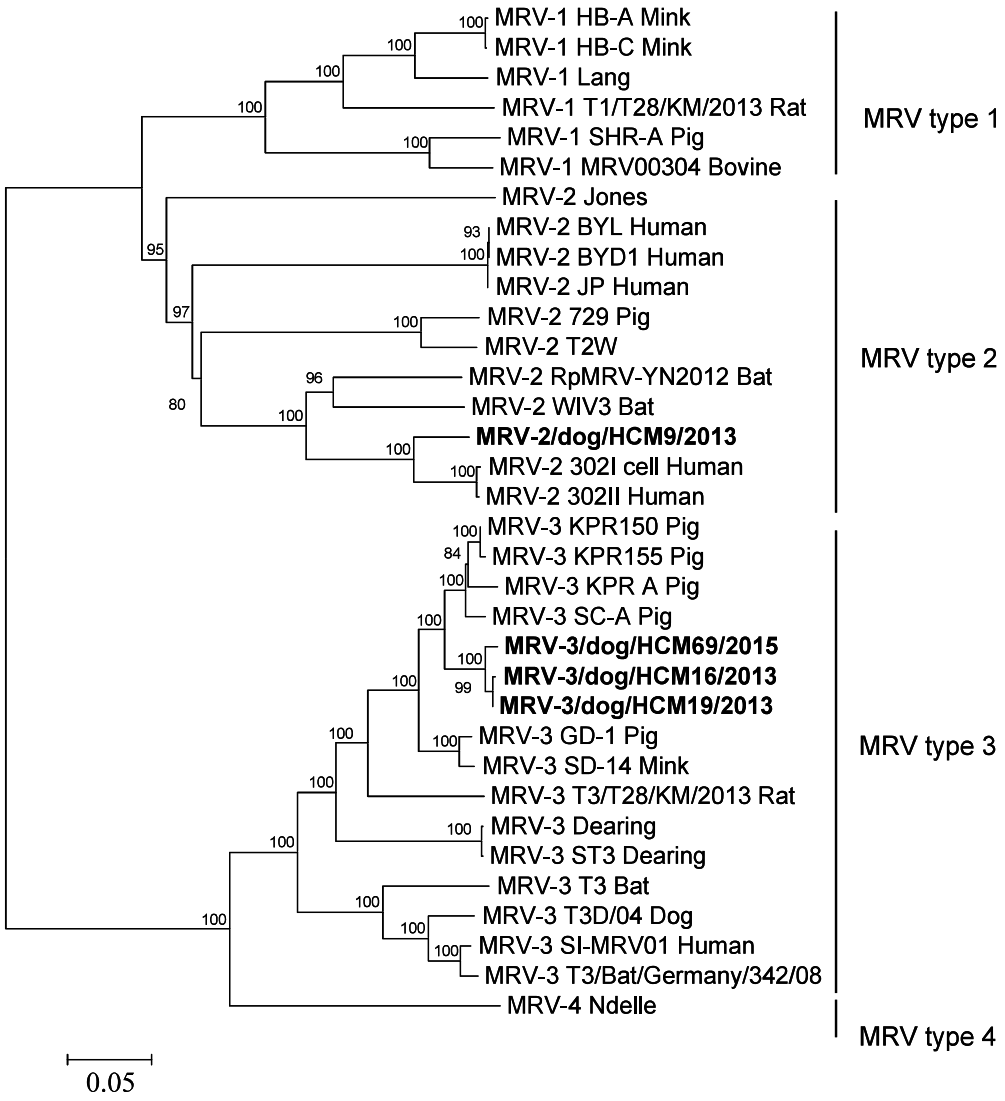

MRV-1 HB-A Mink
MRV-1 HB-C Mink
MRV-1 Lang
MRV-1 T1/T28/KM/2013 Rat
MRV-1 SHR-A Pig
MRV-1 MRV00304 Bovine
MRV type 1
MRV-2 Jones
MRV-2 BYL Human
MRV-2 BYD1 Human
MRV-2 JP Human
MRV-2 729 Pig
MRV-2 T2W
MRV-2 RpMRV-YN2012 Bat
MRV-2 WIV3 Bat
MRV-2/dog/HCM9/2013
MRV-2 302I cell Human
MRV-2 302II Human
MRV type 2
MRV-3 KPR150 Pig
MRV-3 KPR155 Pig
MRV-3 KPR A Pig
MRV-3 SC-A Pig
MRV-3/dog/HCM69/2015
MRV-3/dog/HCM16/2013
MRV-3/dog/HCM19/2013
MRV-3 GD-1 Pig
MRV-3 SD-14 Mink
MRV-3 T3/T28/KM/2013 Rat
MRV-3 Dearing
MRV-3 ST3 Dearing
MRV-3 T3 Bat
MRV-3 T3D/04 Dog
MRV-3 SI-MRV01 Human
MRV-3 T3/Bat/Germany/342/08
MRV type 3
MRV-4 Ndelle
MRV type 4
0.05

Fig.5.2

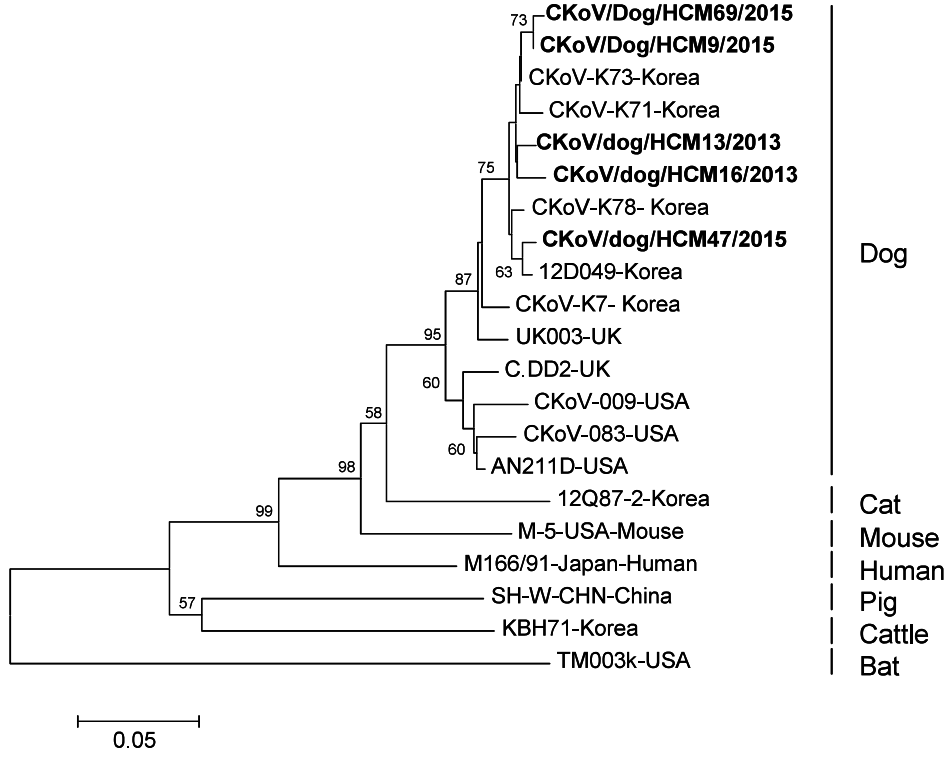
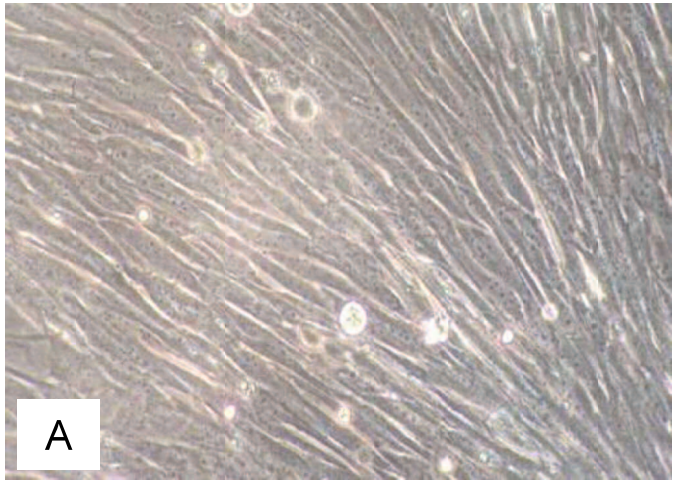
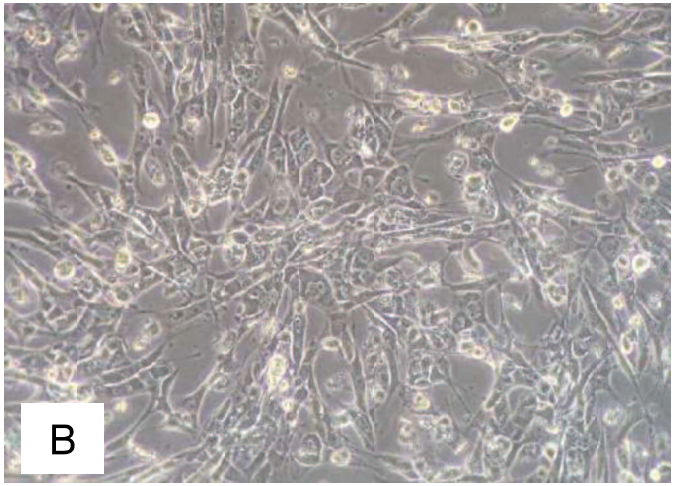

CKoV/Dog/HCM69/2015
CKoV/Dog/HCM9/2015
CKoV-K73-Korea
CKoV-K71-Korea
CKoV/dog/HCM13/2013
CKoV/dog/HCM16/2013
CKoV-K78- Korea
CKoV/dog/HCM47/2015
12D049-Korea
CKoV-K7- Korea
UK003-UK
C.DD2-UK
CKoV-009-USA
CKoV-083-USA
AN211D-USA
12Q87-2-Korea
M-5-USA-Mouse
M166/91-Japan-Human
SH-W-CHN-China
KBH71-Korea
TM003k-USA
73
75
63
87
95
60
58
60
98
99
57
Dog
Cat
Mouse
Human
Pig
Cattle
Bat
0.05

Fig.5.3





6. General conclusion

Enteric viruses cause enteritis in dogs and some of them cause severe diarrheic disease with high mortality and morbidity such as canine distemper virus (CDV) and canine parvovirus (CPV). There were a few reports on CDV and CPV infections in Vietnam, but not on the other enteric viruses such as canine coronavirus (CCoV), canine kobuvirus (CKoV) and mammalian orthoreovirus (MRV). To understand Vietnamese enteric viruses in dogs, we carried out genetic analysis and virus isolation.

In CHAPTER 1, Vietnamese CDVs were characterized. CDV H genes were detected in five domestic Vietnamese dogs with diarrhea and two CDVs were successfully isolated from dogs positive for H genes. The complete genome of one isolate, CDV/dog/HCM/33/140816, was determined. Phylogenetic analysis showed that all Vietnamese CDVs belonged to the Asia-1 genotype. In addition, the H proteins of Vietnamese CDV strains were the most homologous to those of Chinese CDVs (98.4% to 99.3% identity). These results indicated that the Asia-1 genotype of CDV was the predominant genotype circulating among the domestic dog population in Vietnam and that transboundary transmission of CDV has occurred between Vietnam and China.

In CHAPTER 2, Vietnamese CCoVs were characterized. To examine the prevalence of CCoV infection among Vietnamese dogs, 201 serum samples were analyzed by virus-neutralization (VN) test. The results showed that antibodied against CCoV-II were present in 87 dogs (43.3%). To detect genes of CCoV, fecal samples collected from 30 diarrheic and 50 healthy dogs were examinated by RT-PCR, confirming that 2 diarrheic dogs and 5 healthy dogs were positive for CCoV. Nucleotide sequences of *N*-terminal region of S gene indicated that CCoV strains were divided into two subgenotypes, CCoV-IIa and -IIb, respectively. Furthemore, we succeeded in isolation of CCoV/dog/HCM47/2015, the isolate was plaque-purified three times, and 3'-terminal one-third of the genome was analyzed. Interestingly, the plaque-purified

virus had a large deletion in ORF3abc and E genes (1,165 nt), and a short deletion in ORF7b gene (60 nt), suggesting that these regions are not necessary for *in vitro* replication of CCoV. Next, the antigenicity between the isolated CCoV-IIb and the other CCoV-IIa was compared by VN test, revealing that antigenicty of the isolated CCoV is equal or higher than that of the other CCoV. In summary, two subgenotypes of CCoV-II are spreading among Vietnamese dogs. The isolated virus with a large deletion after *in vitro* passage may be useful for the development of vaccine, owing to its antigenicity and efficient viral growth *in vitro*.

In CHAPTER 3, Vietnamese CPVs were characterized. To examine the prevalence of antibody against CPV-2 among Vietnamese dogs, 108 serum samples from unvaccinated dogs were analyzed by VN test. The results showed 101 dogs (93.5%) were positive for anti-CPV antibody. To detect genes of CPV, fecal samples collected from 30 diarrheic and 50 healthy dogs were examined by PCR. The prevalence of anti-CPV antibody in diarrheic dogs (43.3%, 13/30) was significantly higher than that in healthy dogs (4%, 2/50), indicating that CPV was a major pathogen causing diarrhea in domestic dogs. Genotyping of 15 CPV strains showed that CPV-2a (1/15) and CPV-2c (14/15) were circulating in Vietnam. Virus isolation was performed from fecal samples using A72/cSLAM cells, and nine CPV strains were successfully isolated. Phylogenetic analysis based on VP2 gene (1,755 nt) and whole coding region (4,269 nt) indicated that Vietnamese CPV strains were similar to Chinese strains. In conclusion, dominant genotype spreading among Vietnamese dogs changed from CPV2b to CPV-2c.

In CHAPTER 4, Vietnamese MRVs and CKoVs were characterized. The prevalence of MRV in diarrheic dogs (50%, 15/30) was significantly higher than that in healthy dogs (4%, 2/50). Mix-infection with MRV and the other enteric viruses (CDV,

CPV, CKoV) was commonly observed in diarrheic dogs. Four MRVs were isolated from feces, and genetic analysis of MRV S1 genes showed that three Vietnamese MRVs belonged to serotype 3 and one did to serotype 2. Vietnamese serotype 3 and 2 showed the highest identities with Chinese porcine MRV-3 (95.2%-96.1%) and Chinese human MRV-2 (94.6%), respectively. CKoV was detected from three diarrheic (10%) and two healthy dogs (4%). Nucleotide sequence of CKoV showed that Vietnamese CKoV was similar to those in Korea. This is the first report on MRV and CKoV infections in Vietnamese dogs.

In conclusion, we performed comprehensive surveillance of canine enteric viruses in Vietnamese dogs. Interestingly, all Vietnamese viruses were the most similar to those in China, indicating transboundary transmission between Vietnam and China. We succeeded in isolation of two CDVs, one CCoV, nine CPVs and four MRVs. These viruses should be available for development of diagnostic method and vaccine in Vietnam.

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