Prevalence of canine coronavirus and parvovirus infections in dogs with gastroenteritis in Thailand

K. Sakulwira[1], P. Vanapongtipagorn[2], A. Theamboonlers[2], K. Oraveerakul[3], Y. Poovorawan[2]

[1]Department of Anatomy, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand

[2]Viral Hepatitis Research Unit, Department of Pediatrics, Chulalongkorn University & Hospital, Bangkok, Thailand

[3]Division of Virology, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand

ABSTRACT: Canine coronavirus (CCV) and canine parvovirus type 2 (CPV-2) are the causative agents of gastroenteritis in dogs. Seventy fecal samples from dogs with signs of gastroenteritis (vomiting and diarrhea), twenty-five fecal samples from healthy dogs and one CPV-2 vaccine strain were amplified by semi-nested polymerase chain reaction (PCR) and semi-nested reverse transcriptase polymerase chain reaction (RT-PCR), aimed at specifically studying the gene encoding the most abundant capsid protein VP2 of CPV-2 and spike protein of CCV. The specificity of the CCV RT-PCR product was evaluated by sequencing. Positive specimens comprised 44 samples (62.8%) and 9 samples (12.8%) for CPV-2 and CCV, respectively. In nine CCV positive samples, seven displayed co-infection between CCV and CPV-2. Our CCV sequence (AF482001) showed a 94.9% nucleotide identity to CCV reported in GenBank accession number D13096. High prevalence of CCV and CPV-2 infections was found in 1–2 month- and 3–6 month-old dogs, respectively. Molecular biology of these viruses is important primarily for epidemic control and preventive measures.

Keywords: canine coronavirus; canine parvovirus type 2; gastroenteritis; PCR

Canine viral enteritis should be suspected in dogs with an acute onset of vomiting and diarrhea, especially in puppies and where several animals are affected simultaneously. To date, four viruses have been identified as the essential causes of severe enteritis in dogs: Canine Parvovirus, Canine Coronavirus, Canine Rotavirus and Canine Distemper Virus (Pollock and Carmichael, 1983). Electron microscopic (EM) examination of fecal suspensions or isolation in tissue cultures are the most commonly used techniques for diagnosis of the infection in dogs. Recently, polymerase chain reaction (PCR) has increasingly been employed for detection of pathogens, especially when present at very low titers. The PCR is characterized by high sensitivity, specificity, and rapidity, thus becoming widely used for detecting various microorganisms.

Initially, the only prophylactic intervention available against canine parvovirus type 2 (CPV 2) comprised inactivated or live attenuated feline panleukopenia virus vaccines which proved largely ineffective. At a later, vaccines derived from live attenuated CPV-2 become available. Vaccination with an inactivated canine coronavirus (CCV) vaccine can significantly reduce not only viral replication, but the occurrence of clinical disease following a virulent CCV infection (Fulker *et al.*, 1995). In Thailand, the data available on CCV and CPV-2 infection are limited. The objective of the present study is to evaluate the prevalence of CCV and CPV-2 infections in gastroenteritic dogs by using semi-nested RT-PCR and semi-nested PCR to detect CCV RNA and CPV-2 DNA, respectively, in fecal specimens derived from gastroenteritic dogs and healthy dogs.

MATERIAL AND METHODS

Specimens

The samples investigated comprised 70 fecal specimens from domestic dogs with signs of enteritis as and 25 fecal specimens from healthy dogs aged range from 2 months to 1 year brought for the first vaccination to the veterinary clinic, Patumthani province, Thailand between May 1999 and December 2001. Fecal samples were suspended in PBS at a concentration of 10% (w/v or v/v) and centrifuged at 3 000 g at room temperature for 10 min. None of the dogs received any CCV or CPV-2 vaccine previously. The commercially available vaccine (Parvodog Liquide-P vaccine, Cornell strain, Rhone-Merieux Lot no. 80 × 451-06NOV2001) specific for genotype CPV-2 served as a positive control. All specimens were kept in –70°C.

Reverse transcription-polymerase chain reaction detection of CCV RNA

CCV-RNA was extracted using the guanidine method (Cha *et al*., 1991), denatured at 65°C for 5 min, and then reverse-transcribed into cDNA, in a total volume of 20 µl and at 37°C for 1 h, using 50 units MuLV reverse transcriptase (Perkin Elmer). Ten µl RNA, 50 mM KCl, 10 mM Tris-HCl (pH 8), 400 µM dNTP, and 10 units RNAse inhibitor were added to a reaction mixture. CCV RNA was detected by semi-nested RT-PCR using three primers created from the 3′ end of spike gene of CCV strain Insavc-1 (Horsburgh *et al*., 1992). In the first amplification step, 2 µl cDNA sample were amplified in a 50 µl reaction volume containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8), 200 µM dNTP, 1.5 units Taq DNA polymerase (QIAGEN, Germany) and 15 pM each of outer sense primer; CCV1 (located at position 1691: 5′ GGC GTA ACT GAT GGA CCA CG 3′) and outer anti-sense primer; CCV2 (located at position 2451: 5′ CTT GTA CGG GCG GCA ACA TC 3′). PCR conditions consisted of 94°C for 2 min, followed by 40 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 1 min. The condition for the second amplification step were similar to those for the first although the annealing time at 60°C, the inner anti-sense primer; CCV3 (located at position 1887: 5′ GCT CCA CTA GCA CCA GTG G 3′) were used. Ten µl of each amplified DNA samples were loaded on 2% agarose gel and visualized by ultra-violet fluorescence after staining with ethidium bromide.

Polymerase chain reaction detection of CPV-2 DNA

For CPV-2 detection, the DNA was extracted using the alkaline extraction method adjusted for an initial volume of 15 µl. For CPV-2 DNA detection, we amplified the VP2 gene by semi-nested PCR (Sakulwira *et al*., 2001) in an automated thermocycler (Perkin Elmer Cetus, Norwalk, USA). Briefly, 10 µl of the respective DNA sample were added to a reaction mixture containing 1.5 units Taq polymerase (Perkin Elmer Cetus), 250 µM dNTP, primer pair P1 (located at position 3,342: 5′-TCCAGCAGCTATGAGATC-3′) and P2 (located at position 4,588: 5′-GATCTGTTGGTAGCAATAC-3′) for the first amplification round and primer pair P1 and P3 (located at position 4,088: 5′-GATCTGTTGGTAGCAATAC-3′) for the second amplification round, each primer at a 16.5 pM concentration, 5 mM Tris buffer and 0.75 mM $MgCl_2$ at a final volume of 45 µl. The first amplification round consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles comprising a 30 s denaturation step at 94°C, a 2 min annealing step at 50°C and a 2 min extension step at 72°C, each. The amplification was concluded by terminated step, a 10 min elongation step at 72°C. For the second amplification round 1.5 µl of the first PCR product were added to the reaction mixture and amplification was performed in a manner identical to the first round. Ten µl of each amplified DNA sample were loaded on 2% agarose gel stained with ethidium bromide on preparation. Electrophoresis was performed.

We sequenced the CCV-PCR product to confirm and used it further as a positive control. The CCV-PCR product was purified for sequencing using the QIAquick Gel Extraction Kit (Qiagen Inc, Valencia, CA, USA) according to the manufacturer's specifications and subsequently subjected to 2% agarose gel electrophoresis in order to ascertain its purity. DNA was subjected to cycle sequencing using dye-labeled terminators which represents a rapid and convenient method for performing enzymatic extension reactions for subsequent DNA sequencing on the ABIPRISM^{TM}310 Genetic Analyser (Perkin Elmer Cetus, Branchburg, New Jersey, USA). This round

of amplifications was performed according to the manufacturer's specifications using primer pair CCV1 and CCV3 to amplify the particular DNA strand of interest for further sequencing. Cycle sequencing consisted of 25 cycles at 96°C for 10 s (denaturation), 50°C for 5 s (annealing), and 60°C for 4 min (extension). The reaction was concluded by cooling the thermal ramp to 4°C. The extension products were subsequently purified from excess un-incorporated dye terminators by ethanol precipitation, according to the manufacturer's specifications (Perkin Elmer Cetus, ABIPRISM^{TM}310 Genetic Analyser). For all the sub-sequent steps we referred to the ABIPRISM^{TM}310 Genetic Analyser user's manual (Perkin Elmer Cetus, Branchburg, New Jersey, USA).

The product band of 197 bp of CCV and 747 bp of CPV-2 were visualized on a UV-light box were shown in Figures 1 and 2, respectively. CCV sequence analysis of the RT-PCR products was sequenced using the outer sense primer; CCV1 and confirmed by the inner anti-sense primer; CCV3. Our CCV sequence (accession number AF482001) had 94.9% nucleotide identity (CLUSTAL X alignment program) to CCV strain Insavc-1 sequence (accession number D13096).

RESULTS

Seventy fecal samples from dogs with diarrhea, 1 month-5 years old, thirty-seven males, twenty-six females and seven unrecorded sex were included in these study. None of the specimens originating from healthy dogs provided evidence of CCV RNA or CPV-2 DNA. In contrast, 9 (12.8%) and 44 (62.8%) out of the 70 samples from gastroenteritic dogs proved positive for CCV RNA and CPV-2 DNA, respectively, by semi-nested RT-PCR and semi-nested PCR (Table 1). In forty-four CPV-2 DNA positive samples, there were twenty-three males, eighteen females and three of unrecorded sex. In nine CCV-RNA positive samples, there were six males and three females. Seven showed co-infection between CCV and CPV-2. One showed only CCV infection. High prevalence of CCV and CPV-2 infections was found in 1–2 month- and 3–6 month-old dogs, respectively. None of the dogs

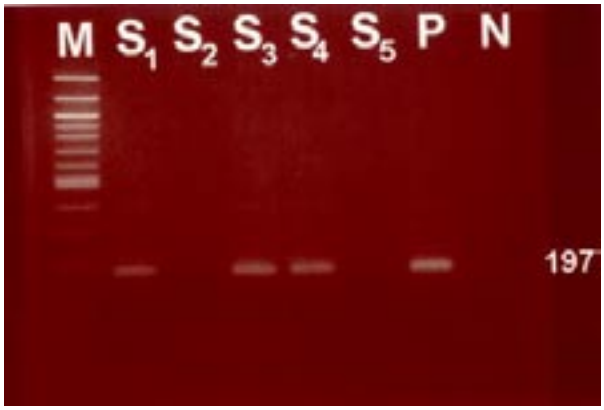

Figure 1. PCR products (197 bp) of CCV. M = 100-bp marker; P = PCR positive control; N = PCR negative control; S1–5 = PCR samples 1–5

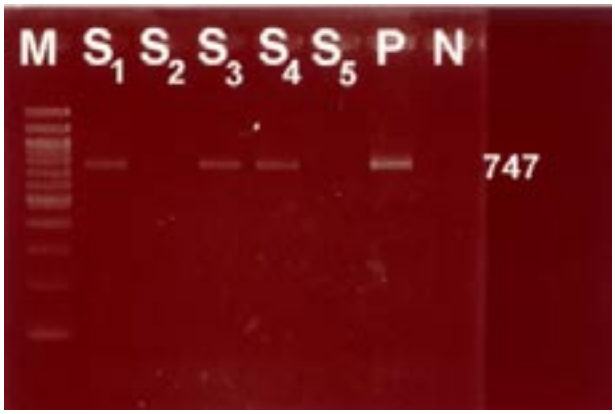

Figure 2. PCR products (747 bp) of CPV-2. M = 100-bp marker; P = PCR positive control; N = PCR negative control; S1–5 = PCR samples 1–5

Table 1. CCV RNA and CPV-2 DNA detected in gastroenteritic dogs of different age groups

Age	Number of samples	CCV positive	CPV-2 positive	Co-infection
1–2 months	17	5 (29.4%)	12 (70.5%)	3 (17.6%)
3–6 months	34	3 (8.8%)	25 (73.5%)	3 (8.8%)
>6 months	5	0 (0)	0 (0)	0 (0)
Unknown	14	1 (7.1%)	(50.0%)	1 (7.1%)

above 6 months of age contained CCV RNA or CPV-2 DNA in their fecal samples (Table 1).

DISCUSSION

CCV and CPV-2 are recognized as important pathogens responsible for severe enteritis in dogs. There are several methods used for detection of these viruses: detection of virus in fecal specimens, serology and histopathology with immunochemistry (for CPV). The nested RT-PCR assay was developed and tested with feline infectious peritonitis virus (FIPV), feline enteric coronavirus (FECV), CCV and transmissible gastroenteritis virus (TGEV) (Gamble *et al*., 1997) and found to be highly sensitive and specific (Pratelli *et al*., 1999). The results of nested RT-PCR assay for the diagnosis of CCV revealed an apparent high sensitivity when compared to virus isolation and EM (Pratelli *et al*., 2000). PCR was shown to detect virus even at a concentration as low as about 10 infectious particles per reaction, corresponding to a virus titer of about 10^3 PFU/ml of unprocessed fecal material (Schunck *et al*., 1995). This sensitivity is good agreement with results of a published study by another group using different primers, pretreatment protocols and PCR conditions (Uwatoko *et al*., 1995). They reported PCR sensitivities after gel chromatography and boiling pretreatment of about 10–100 PFU parvovirus per reaction.

Epidemiology of canine enteritic infections was studied in Japan (Mochizuki *et al*., 2001). Most frequently detected in both diarrheal and normal feces was CCV (55.4%). CPV-2 was specifically associated with diarrheal cases. Bandai *et al*. (1999) elucidated the prevalence of CCV infections in dogs in Japan by using nested RT-PCR on rectal swabs of 100 diarrheic dogs presenting in a veterinary clinic. Evidence of CCV was found in 16% of these specimens. Tingpalapong *et al*. (1982) reported that dogs in Thailand were infected with CPV-2 and CCV by hemagglutination inhibition and neutralizing antibody tests, respectively, and these viruses were most likely the causes of viral enteritis.

In the present study, we detected CCV RNA and CPV-2 DNA by semi-nested RT-PCR and semi-nested PCR. The percentage of enteric dogs testing CCV and CPV-2 positive was found to amount to 9/70 (12.8%) and 44/70 (62.8%), respectively. It was interesting that there was no evidence of either CCV or CPV-2 among the healthy dogs in our study. However, these findings were based on limited population area studied at a single veterinary clinic. Therefore, further studies are needed to evaluate these findings in other parts of Thailand. Mochizuki *et al*. (1993) detected CPV-2 DNA from fecal samples of dogs with diarrhea by nested PCR. They found CPV-2 DNA in 22 out of 59 (37.3%) fecal samples. Schunck *et al*. (1995) detected CPV-2 DNA from fecal specimens derived from enteritic dogs by single-round PCR. Using these method, they established the presence of CPV-2 DNA in 54 out of 65 (83.1%) samples tested. Only a few amino acids within the capsid protein gene (in particular residues 93 and 323) control the canine host-range and antigenic difference between feline panleukopenia and canine parvovirus. Comparison of CPV-2 and CPV-2a/-2b antigenicity shows amino acid difference at 300 and 305 (Truyen *et al*., 1995). Based on this, we were able to differentiate between vaccine (CPV-2) and field viruses (CPV-2a or CPV-2b) by using restriction fragment length polymorphism (Sakulwira *et al*., 2001). The incidence of virus may be truly different, owing to differences between the density and social contact of the dog populations investigated. However, the methods used to demonstrate the virus differ, and the efficiency of detection may vary.

Our principal interest was to use semi-nested RT-PCR and semi-nested PCR as methods to rapidly differentiate CCV or CPV-2 infections from other canine enteric pathogens, which can cause similar clinical illnesses. Definitive diagnosis is important primarily for epidemic control and preventive measures.

Acknowledgement

The authors would like to thank the staff of Viral Hepatitis Research Unit, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, for their assistance in the molecular laboratory and to Faculty of Veterinary Medicine, Chulalongkorn University for collecting the specimens. We also would like to thank the Thailand Research Fund, Senior Research Scholar and the Molecular Research Project, Faculty of Medicine, Chulalongkorn University for supporting this research.

REFERENCES

Bandai C., Ishiguro S., Masuya N., Hohdatsu T., Mochizuki M. (1999): Canine coronavirus infections in

Japan: virological and epidemiological aspects. J. Vet. Med. Sci., *61*, 731–736.

Cha T.A., Kolberg J., Irvine B., Stempien M., Beall E., Yano M., Choo O.L., Houghton M., Kuo G., Han J.H. (1991): Use of a signature nucleotide sequence of hepatitis C virus for detection of viral RNA in human serum and plasma. J. Clin. Microbiol., *9*, 2528–2534.

Fulker R., Wasmoen T., Atchison R., Chu H.J., Acree W. (1995): Efficacy of an inactivated vaccine against clinical disease caused by canine coronavirus. Adv. Exp. Med. Biol., *380*, 229–234.

Gamble D.A., Lobbiani A., Gramegna M., Moore L.E., Colucci G. (1997): Development of a nested PCR assay for detection of feline infectious peritonitis virus in clinical specimens. J. Clin. Microbiol., *35*, 673–675.

Horsburgh B.C., Brierley I., Brown T.D. (1992): Analysis of a 9.6 kb sequence from the 3′ end of canine coronavirus genomic RNA. J. Gen. Virol., *73*, 2849–2862.

Mochizuki M., San Gabriel M.C., Nakatani H., Yoshida M. (1993): Comparison of polymerase chain reaction with virus isolation and hemagglutination assays for the detection of canine parvoviruses in fecal specimens. Res. Vet. Sci., *55*, 60–63.

Mochizuki M., Hashimoto M., Ishida T. (2001): Recent epidemiological status of canine viral enteric infections and Giardia infection in Japan. J. Vet. Med. Sci., *63*, 573–575.

Pollock R.V.H., Carmichael L.E. (1983): Canine viral enteritis. Vet. Clin. North Am. Small Anim. Pract., *13*, 551–566.

Pratelli A., Tempesta M., Greco G., Martella V., Buonavoglia C. (1999): Development of a nested PCR assay for the detection of canine coronavirus. J. Virol. Methods, *80*, 11–15.

Pratelli A., Buonavoglia D., Martella V., Tempesta M., Lavazza A., Buonavoglia C. (2000): Diagnosis of canine coronavirus infection using nested-PCR. J. Virol. Methods, *84*, 91–94.

Sakulwira K., Oraveerakul K., Poovorawan Y. (2001): Detection and genotyping of canine parvovirus in enteritic dogs by PCR and RFLP. Science Asia, *27*, 143–147.

Schunck B., Kraft W., Truyen U. (1995): A simple touchdown polymerase chain reaction for the detection of canine parvovirus and feline panleukopenia virus in feces. J. Virol. Methods, *55*, 427–433.

Tingpalapong M., Whitmire R.E., Watts D.M., Burke D.S., Binn L.N., Tesaprateep T., Laungtongkum S., Marchwicki R.H. (1982): Epizootic of viral enteritis in dogs in Thailand. Am. J. Vet. Res., *43*, 1687–1690.

Truyen U., Gruenberg A., Chang S.F., Obermaier B., Veijalainen P., Parrish C.R. (1995): Evolution of the feline-subgroup parvoviruses and the control of canine host range in vivo. J. Virol., *69*, 4702–4710.

Uwatoko K., Sunairi M., Nakajima M., Yamaura K. (1995): Rapid method utilizing the polymerase chain reaction for detection of canine parvovirus in feces of diarrheic dogs. Vet. Microbiol., *43*, 315–323.

Received: 03–02–26
Accepted after corrections: 03–05–09

Corresponding Author

Prof. Yong Poovorawan, Viral Hepatitis Research Unit, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University & Hospital Bangkok 10330, Thailand
Tel. +66 2 256 49 09, fax +66 2 256 49 29, e-mail: Yong.P@chula.ac.th