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Evaluation for Serological Patterns and Fecal Viral Shedding by Hemagglutination Inhibition Test and Real-time PCR in Korean CPV-2 isolates

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Abstract : We evaluated the patterns of serology and fecal viral shedding for any differences by hemagglutination inhibition (HI) and real-time PCR on Korean CPV-2 isolates (CPV-2a-I, CPV-2a-V and CPV-2b). We successfully detected fecal viral shedding from samples extracted 2-3 d.p.i., regardless of the onset of clinical signs. In addition, the pattern of viral shedding differed depending on the CPV-2 isolates used for inoculation. We also observed differences in the serological pattern that was also depended on the CPV-2 isolates inoculated. The onset and amount of fecal viral shedding were not correlated with the level of antibody titers in this study. Our study is a valuable resource for understanding the different pathobiology of the CPV-2 isolates and the correlation between the patterns of serum antibody titer and fecal viral shedding.

Key words **:** canine parvovirus, real-time PCR, hemagglutination inhibition (HI), Korea.

Introduction

Canine parvovirus type 2 (CPV-2), is a subgroup of the genus *Parvovirus*, the major pathogen of acute hemorrhagic gastroenteritis in puppies (1). CPV-2 is antigenically and genetically related to the feline panleukopenia virus (FPLV).

There are only a few amino acid differences between CPV-2 and FPLV (14,19). CPV-2 was first reported in the late 1970s (2), and since then, has been replaced by two new antigenic variants, type 2a and 2b (17,20). Recently, a third antigenic variant, CPV-2c has been reported in several countries (3,5,16).

Despite an active protection program, canine parvovirus 2 (CPV-2) is still found throughout Korea, where CPV-2 remains the most common pathogen for hemorrhagic enteritis in pups (10). Recently, CPV type 2a has been identified as the most prevalent isolate of CPV-2 and to a lesser extent, CPV type 2b in Korea (11,15). In a recent epidemiological study, we found CPV-2a was further subdivided into CPV-2a-I to CPV-2a-V, based on amino acid substitutions in the VP2 gene (10). Furthermore, we found that CPV-2a-I and CPV-2a-V were the most prevalent variants in Korea (10).

Previously, ELISA and hemagglutination (HA) tests were the methods of choice used to detect viruses in the feces of CPV-2 infected animals (13,21). These methods were replaced by a polymerase chain reaction (PCR) method due to its higher sensitivity (3,18). Since then, real-time PCR (or quantitative PCR) is preferred due to a higher sensitivity and accuracy compared to the previous methods used (6). Therefore, CPV-2 infected animals with a very low titer can still be detected.

The aim of our study was to evaluate the differences in the patterns of serology and of fecal viral shedding by using an hemagglutination inhibition (HI) test and real-time PCR on Korean CPV-2 isolates (CPV-2a-I, CPV-2a-V and CPV-2b).

Materials and Methods

Subjects

Prior to the onset of our investigation, we obtained the approval of the animal ethics committee of Kangwon National University. This animal testing program (including animal care, euthanasia and disposal of dead animals) strictly adhered to the guidelines of the National Research Council of the Korea. Fifteen mixed breed puppies aged 6-8 weeks were screened and selected based on having a negative CPV response using a commercially available test kit (Canine Parvovirus antigen kits, Idexx, USA). Only dogs with no protective maternal antibody against CPV-2, confirmed by the HI test were enrolled in this study. The animals selected were further subdivided into three groups of 5. The animals were housed individually and fed twice daily on a commercial meat based diet. Water was freely available at all times. Prior

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to inoculation, all animals were tested for no pre-existing CPV-2 infection, which was confirmed from the feces of all animals by evidence of the lack of viral shedding, using a PCR method previously described (10). One milliliter (10^6TCID$_{50}$) of each field virus (CPV-2a-I, CPV-2a-V or CPV-2b), all of the third passage, were used to inoculate each group of animals oronasally.

After viral inoculation, fecal samples were collected daily for 15 days for confirmation of CPV-2 infection by real-time PCR analysis. Serum samples were also collected everyday post-infection for evaluating the antibody response to CPV-2 using an HI test.

Viruses

All CPV-2 variants were obtained from naturally infected dogs. These were propagated in a canine fibroma cell line A72 and grown (for 72h at 37 °C in 5 % CO_2) in Dulbeco's modified Eagle's medium supplemented with 10% fetal bovine serum. CPV-2a-I is found only in Korea with the amino acid substitution Asp413Asn, Ile418Thr and Thr440Ala in the VP2 gene of the CPV-2a strain (Table 1). CPV-2a-V has a Tyr324Ile substitution (Table 1), found in Korea and in other countries such as Italy, Vietnam, Taiwan and USA. CPV-2b is found worldwide and has a Asn426Asp substitution in the CPV-2a strain (Table 1).

Real-time PCR assay

DNA for real-time PCR analysis was extracted from feces using an AccuPrep® Genomic DNA Extraction Kit (Bioneer Corporation, Korea). The DNA samples were diluted 1:10 in distilled water, to reduce the levels of residual inhibitors of DNA polymerase activity which is detrimental to the assay. Real-time PCR was performed on duplicates of the CPV standard and DNA samples simultaneously as described previously (6), in a Rotor-GeneTM 3000 (Corbett research, Australia). The setup of each reaction consisted of a 25 μl PCR reaction containing 12.5 μl of master mix, 600 nM of primers CPV-for and CPV-Rev, 200 nM of CPV-Pb probe (Table 2), and 10 μl of DNA. Serial 10-fold dilutions (representing from 10^9 to 10^2 DNA copies/10 μl of standard DNA) of plasmid containing the nearly full-length genome of CPV-2 were used to generate a standard curve. For a precise quantification of CPV-2 DNA in all fecal samples, fixed amount of universal internal control were added into fecal samples. For preparing universal internal control, pGEM-cGATA4 plasmid was made and purified as described in Lee et al. (12).

The 5×10^6 copies/ μl of pGEM-cGATA4 plasmid DNA was added into each in 1 ml of fecal suspension. The CPV-2 and pGEM-cGATA4 real-time PCR assays were carried out as a duplex PCR in a single tube, with the condition as follows: the thermal conditions were for a duplex real-time PCR assay: activation of *Taq* DNA polymerase at 95 °C for 10 min and 40 cycles consisting of denaturation at 95 °C for 10 min and 40 cycles consisting of denaturation at 95 °C for 15 sec, primer annealing at 52 °C for 30 sec and extension at 59 ºC for 1 min. GATA-Ex3F (5'-cagcacttgccttctatttg-3') and GATA-Ex3R (5'-ctgccctccccctgcttgtgt-3') primers were used for the amplification of internal control. Fluorescence (F1 filter) was

Table 1. Amino acid sequence variations of the VP2 gene of canine parvovirus type 2 in reference strains and Korean isolates used in this study.

Amino acid position	Reference strains				Korean isolates		
	CPV-2	CPV-2a	CPV-2b	CPV-2c	CPV-2a-I	CPV-2a-V	CPV-2b
87	Met	Leu	Leu	Leu	Leu	Leu	Leu
101	Ile	Thr	Thr	Thr	Thr	Thr	Thr
300	Ala	Gly	Gly	Gly	Gly	Gly	Gly
305	Asp	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr
324	Tyr	-*	-	-	-	Ile	-
413	Asp	-	-	-	Asn	-	-
418	Ile	-	-	-	Thr	-	-
426	Asn	-	Asp	Glu	-	-	Asp
440	Thr	-	-	-	Ala	-	-
555	Val	-	-	-	-	-	-

* (-) means no difference from CPV-2 reference strain.

Table 2. Sequence and position of oligonucleotides used in the study.

Primer /probe	Sequence 5' to 3'	Sense	Amplicon size (bp)
CPV-For	AAACAGGAATTAACTATACTAATATATTTA	+	93
CPV-Rev	AAATTTGACCATTTGGATAAACT	-	
CPV-Pb	FAM-TGGTCCTTTAACTGCATTAAATAATGTACC-TAMRA	+	

FAM = 6-carboxyfluorescein; TAMRA = 6-carboxytetramethylrhodamine.

measured (excitement: 488 nm, emission: 560 nm) at the end of each extension step. To verify the presence of the desired PCR product, melting analysis was conducted. The data evaluation was accomplished using the Light Cycler Data Analysis software (Corbett research, Australia).

Hemagglutination inhibition (HI) test

The HI test was performed on the serum as described by Carmichael et al. (4), with minor modifications. The tests were performed at 4 ºC in 96 well V-plates, using 10 hemagglutination units of CPV-2 antigen and 1 % pig erythrocytes. Each serum sample was diluted two-fold in PBS starting from 1:10. The HI titer was indicated as the highest serum dilution completely inhibiting viral hemagglutination.

Results

Fecal shedding

The median titers of CPV-2 DNA from the Korean isolates detected by real-time PCR of feces from each group were shown in Fig 1. Very low titers of CPV-2 DNA were shown on the first day after inoculation for all groups. In all groups, fecal shedding was confirmed by the real-time PCR assay. However, detecting the earliest and the peak time of CPV-2 infection differed between groups. Viral shedding was detected at 2 d.p.i. for the CPV-2b group, and a peak time at 3 d.p.i. Fecal shedding for CPV-2b decreased during 4 to 7 d.p.i. After 7 d.p.i, viruses in the feces were found to have a very low titer (Fig 1). On the other hand, viral shedding was also detected at 2 d.p.i, with a peak time at 5 d.p.i for the CPV-2a-I variant. Although the amount of fecal viral shedding increased gradually, the duration and severity of viral shedding for the CPV-2a-I variant were the longest and highest among the Korean CPV-2 isolates (Fig 1). Viral shedding was detected at 3 d.p.i for the CPV-2a-V group, but decreased at 8 d.p.i (Fig 1).

Serology

Serological analyses on the Korean CPV-2 isolates were evaluated by the HI test (Fig 2). The antibody titers for the CPV-2a-I and CPV-2a-V groups slowly increased reaching the protective level at 8 d.p.i., and continued to increase till

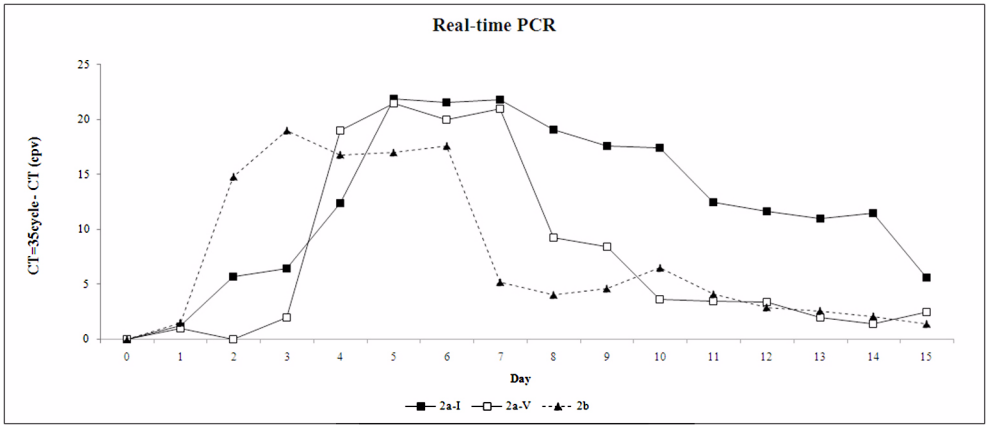

Fig 1. Patterns of fecal viral shedding in Korean CPV-2 isolates evaluated by real-time PCR. Values are expressed as median titers of viral-DNA copy numbers/mg feces of each group.

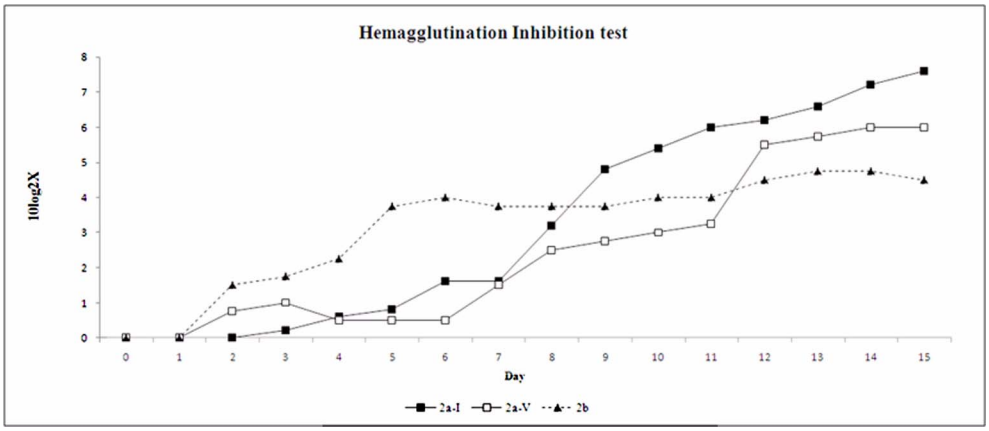

Fig 2. Patterns of serological changes in dogs after inoculating different type of Korean CPV-2 isolates. Values are geometric means of HI titers of each group.

the end of the study. In contrast, the antibody titer for the CPV-2b group increased at a faster rate reaching the protective level at 5 d.p.i. Unlike the CPV-2a groups, the antibody titer for the CPV-2b group did not continue to increase or maintain the peak level (Fig 2).

Discussion

Even now the CPV-2 virus, which is prevalent in Korea, is easily transmitted via oronasal infection and is especially fatal in puppies (10). ELISA and HA test which were techniques used in the past have low sensitivity (13,21), and PCR was put to general use (3,18), it cannot quantify the amount of viruses. The immuno-fluorescence antibody (IFA) test which uses cell culture to titrate the amount of viruses, it consumes a lot of time and the process is complicate. However, because real-time PCR has high sensitivity and can relatively quantify the amount of viruses, it can diagnose CPV-2 rapidly and accurately (6).

In this study, fecal shedding was confirmed by real-time PCR assay from each group. We found very low titers of CPV-2 DNA on the first day after inoculation for all groups. The observed early detection of CPV-2 in the fecal samples might be due to the shedding of residual challenge viruses as reported previously (7). We also found a fecal viral shedding in all groups. According to literatures, viral shedding can be detected up to 7 d.p.i by the HA test and 12 d.p.i by virus isolation in cell cultures (4,18). However, it can be detected by 5 d.p.i. using the real-time PCR assay (8). In this study, fecal shedding of the Korean isolates were detected from 2-3 d.p.i. After inoculation, CPV-2b was the first to be detected, which peaked at 3 d.p.i. whereas CPV-2a-I and CPV-2a-V peaked at 5 d.p.i. The observed rapid increase in fecal shedding for CPV-2b showed was barely detectable after 7 d.p.i.

In this study, the antibody titer in the CPV-2b group was observed to rapidly increased reaching the protective level faster than the other isolates. Since fecal viral shedding are affected by level of the host antibody (9), the earliest increase and steepest decrease in the amount of fecal viral shedding as observed in the CPV-2b group, might be related to the different immunogenicity of each CPV-2 variant. However, although the earliest detection of antibody and the level of antibody titer maintained were similar in the CPV-2a-I and CPV-2a-V groups (most prevalent strains in Korea), the patterns of fecal viral shedding in these two CPV-2a variants were quite different. This suggests that there are other factors that can influence the amount of fecal viral shedding.

According to the literature, the incubation period of CPV-2 infection is around 3-10 days and clinical signs occur after the incubation period (1). Fecal viral shedding is generally observed after the clinical signs are obvious (1). However, in this study, pups infected with CPV-2 isolates were found to shed viruses 2-3 days after infection, regardless of the appearance of clinical signs. This finding is in stark contrast to earlier studies (1). However, the early detection of fecal viral shedding might be due to the superior sensitivity of the real-time PCR assay, rather than due to difference in pathogenicity among the CVP-2 variants.

In conclusion, this study evaluated the differences in the patterns of serology and fecal viral shedding using the HI test and real-time PCR on Korean CPV-2 isolates (CPV-2a-I, CPV-2a-V and CPV-2b). Our study found that fecal viral shedding was detected at 2-3 d.p.i., regardless of the onset of clinical sings. In addition, the patterns of viral shedding differed depending on the CPV-2 isolate inoculated. Although the earliest increase in antibody titer was observed in the CPV-2b group, higher antibody titers were maintained in the CPV-2a groups. There patterns of serology and fecal viral shedding observed in our study was not consistent to that observed in earlier studies. Due to the lack of studies that address the pattern of fecal viral shedding and immunogenicity among CPV-2 variants, our findings are a valuable resource for understanding the different pathobiology of CPV-2 variants and the correlation between patterns of serum antibody titer and fecal viral shedding. However, our study is also limited, in that we did not include the original CPV-2 in our study. Furthermore, our findings were from a study performed on dogs that were experimentally infected.

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