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Detection and Genotyping of Canine Parvovirus in Enteritic Dogs by PCR and RFLP

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ABSTRACT Canine Parvovirus (CPV), a member of the genus *Autonomous Parvovirus*, is a non-enveloped, single-stranded DNA virus of approximately 5 kb. The virus was first described in 1978, with the original isolate being termed CPV type 2 (CPV-2). In 1979, a variant CPV strain designated CPV type 2a (CPV–2a) started to become widespread, followed by a further antigenic variant designated CPV type 2b (CPV-2b), which emerged in 1984. In due course, CPV-2b replaced CPV-2a. The vaccines presently in use are CPV-2 strain specific. Hence, the objective of the present study was to detect and genotype CPV by polymerase chain reaction (PCR), using primer sequences derived from the conserved VP2 region of the genome, and to subsequent restriction fragment length polymorphism (RFLP) analysis of the PCR product. The RFLP analysis employed the endonucleases *Rsa* I and *Hph* I in order to differentiate the CPV-2 antigenic variants and establish their distribution in Thailand. We investigated 55 fecal samples from dogs with signs of enteritis, 55 samples from healthy dogs and CPV-2 strain genotype vaccine. Thirty-four out of the 55 specimens (61.8%) from dogs with enteritis were found to be CPV DNA positive. None of the specimens from healthy dogs provided evidence of CPV DNA. After establishing the difference between wild and vaccine strains using RFLP, we found that all virus strains in our study were either CPV-2a or CPV-2b type, which differed from the vaccine strain (CPV-2). Molecular characterization and CPV typing are crucial in epidemiological studies for future prevention and control of the disease.

KEYWORDS: canine parvovirus, enteritis, PCR, RFLP, genotyping.

INTRODUCTION

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. Canine Parvovirus has been found to be responsible for approximately 27% of canine diarrhea.[1] The family *Parvoviridae* comprises two subfamilies: *Parvovirinae*, which infects vertebrates and is classified into three genera, *Parvovirus*, *Erythrovirus* and *Dependovirus*; and *Densovirinae*, which infects insects and is further classified into three genera, *Densovirus*, *Iteravirus* and *Contravirus*.[2,3] Parvovirus is among the smallest animal DNA viruses, with the virion exhibiting a diameter of between 18 and 26 nm. The genome is comprised of single-stranded DNA of approximately 5,000 bases encoding two structural (VP1 and VP2) and two non-structural (NS1 and NS2) proteins.[4]

CPV-2 emerged as a novel pathogen of dogs in 1978, with outbreaks leading to myocarditis and hemorrhagic gastroenteritis among puppies in Europe and North America.[5-8] Based on the close genetic and, particularly, antigenic relationship, it has been proposed that CPV-2 may have originated by genetic mutation in a wildlife host receptive to one of the feline panleukopenia virus (FPV) -like parvoviruses infected carnivores.[9,10] There is approximately 98% DNA sequence identity between CPV-2, FPV and mink enteritis virus (MEV).[9] However, these viruses can be differentiated on the basis of specific monoclonal antibodies,[11-13] and the pH dependence of hemagglutination. CPV agglutinates rhesus macaque or pig erythrocytes over a broad pH range (pH 6.0 - 8.0), whereas FPV isolates agglutinate these erythrocytes only at slightly acidic condition (pH< 6.8).[14-16]

The first CPV-2a isolates were reported in 1979, whereas CPV-2b variants were not reported until 1984.[17,18] The CPV-2a and CPV-2b subsequently

replaced the CPV-2 on a global scale. As opposed to the original CPV-2, these new antigenic variants have been capable of replicating, as well as causing disease, in cats.[10,19] Genetically, these new types differ from the original one by four to six alterations in the gene coding for the capsid protein.

Parvoviruses display tropism for tissues containing actively dividing cells and replicate in lymphoid cells, as well as in rapidly dividing epithelial cells of the small intestine. For several days after infection, high titers of progeny virus are shed in feces and transmitted by the fecal-oral route to susceptible hosts. Initially, the only prophylactic intervention available against canine parvovirus was comprised of inactivated or live attenuated feline panleukopenia virus vaccines, which proved largely ineffective. At a later stage, vaccines derived from live attenuated canine parvovirus became available; however, the difficulties of eliciting protection in the presence of maternally derived antibodies still remained.[11] The vaccines commercially available at present exhibit CPV-2 specificity.

Laboratory diagnosis of CPV is performed by demonstrating the presence of virus in feces. Although various laboratories have routinely applied the fecal HA/HI assays, problems may arise due to other microbes, cytotoxic substances, or non-specific agglutinin interfering with these assays. Recently, polymerase chain reaction (PCR) has increasingly been employed for detection of pathogens, especially when present at very low titers. Employing this technique, primer-directed enzymatic amplification of specific DNA sequences can be accomplished[20] with the primers preferentially designed to anneal to highly conserved regions of the DNA sequence under investigation; in our case, the gene coding for the capsid protein VP2. PCR is characterized by high sensitivity, specificity, and rapidity, and has thus become widely used for detecting various microorganisms.[21-23] In Thailand, the data available on CPV infection are limited. The objective of the present study is therefore to apply PCR to detect CPV DNA in fecal specimens derived from enteritic dogs and healthy dogs. Furthermore, restriction fragment length polymorphism (RFLP) analysis was performed with the aim of differentiating between wild type and CPV-2 derived vaccine strains.

Materials and Methods

Population Study

The samples investigated consisted of 55 fecal specimens from dogs with signs of enteritis and 55 fecal specimens from healthy dogs brought for the first vaccination to the veterinary clinic, Patumthani Province, between May 1999 and February 2000. There were 64 males and 46 females aged from 1 month to 5 years. None of the dogs had received any CPV vaccine previously. The commercially available vaccine (Parvodog Liquide-P vaccine, Rhone-Merieux Lot no. 80x451-06NOV2001), specific for genotype CPV-2, served as a positive control. All specimens were kept in -70°C.

Laboratory Method

DNA Extraction

For CPV isolation, fecal samples were suspended in PBS at a concentration of 10% (w/v or v/v) and centrifuged at 3000 g at room temperature for 10 min. The DNA was isolated using the alkaline extraction method [24] adjusted for an initial volume of 15 μl.

CPV-DNA Detection

For CPV-DNA detection, we amplified the VP2 gene by semi-nested PCR 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 U of Taq polymerase (Perkin Elmer Cetus), 250 μM each of the four deoxynucleotide triphosphates (Promega), 16.5 pmoles of primer pair: P1 (5'-TCCAGCAGC TATGAGATC-3'; nt no. 3,342-3,360) and P2 (5'-GATCTGTTGGTAGCAATAC-3'; nt no. 4,570-4,588) for the first amplification round and P1 and P3 (5'-GATCTGTTGGTAGCAATAC-3'; nt no. 4,070-4,088) for the second amplification round, 5 mM Tris buffer and 0.75 mM $MgCl_2$ in a final volume of 45 μl. The first amplification round consisted of an initial denaturation step at 94°C for 5 minutes, followed by 30 cycles comprised of a 30 second denaturation step at 94°C, a 2 minute annealing step at 50°C and a 2 minute extension step at 72°C, each. The amplification was concluded by a 10 minute 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 microliters of each amplified DNA sample were loaded onto a 2% agarose gel stained with ethidium bromide on preparation. Electrophoresis was performed at 100 V for 40 minutes.

CPV Typing by RFLP

Canine parvovirus sequences were derived from Genbank, and accession numbers are M19296, U72695 and U72696 for CPV-2, CPV-2a and CPV-2b, respectively. The CLUSTAL X program (NCBI) was used for compare the variant sequences of CPV-2, CPV-2a and CPV-2b from nt 3280 to 4540 and it was found that CPV-2 differs from CPV-2a and CPV-2b by two nucleotides (nt 3685;C to G and 3699;G to T).[18] The Webcutter, Vers. 2 (www.ccsi.com/firstmarket/cutter) was applied for selecting the restriction endonucleases most suitable for genotype-specific cleavage. Accordingly, *Rsa* I (which cuts at nt 3685) and *Hph* I (which cuts at nt 3699) were selected in order to distinguish CPV-2 from CPV-2a and CPV-2b.

Statistical analysis

The percentage of CPV infection is calculated by dividing the number of CPV DNA positive by number of samples. Statistical significance ($p = 0.05$) was calculated by the Chi-Square test.

Results

The product band of 747 base pairs (uncleaved PCR product) was visualized on a UV-light box (Fig 1 and 2). None of the specimens originating from healthy dogs provided evidence of CPV DNA. In contrast, 34 out of the 55 samples (61.8%) from enteritic dogs proved positive for CPV DNA by semi-nested PCR (Table 1). None of the dogs above 6 months of age contained CPV DNA in their fecal samples (Table 1).

Contrasting the apparent influence of age, neither sex nor breed appeared to play any role as to the prevalence of CPV infection ($p>0.05$). In order to differentiate between CPV-2 (vaccine strain) and CPV-2a, CPV-2b (wild strains), all specimens were subsequently subjected to restriction fragment length polymorphism (RFLP) analysis. Digestion by *Rsa* I produced a characteristic pattern distinguishing the vaccine strain CPV-2 from either wild strain CPV-2a or CPV-2b. Similarly, cleavage by *Hph* I provided a specific differentiation pattern. The characteristic product sizes of vaccine strain versus wild type obtained with either enzyme are shown in Fig 1 and 2. All the samples derived from dogs with enteritis were proved to harbor the wild strains CPV-2a and/or CPV-2b.

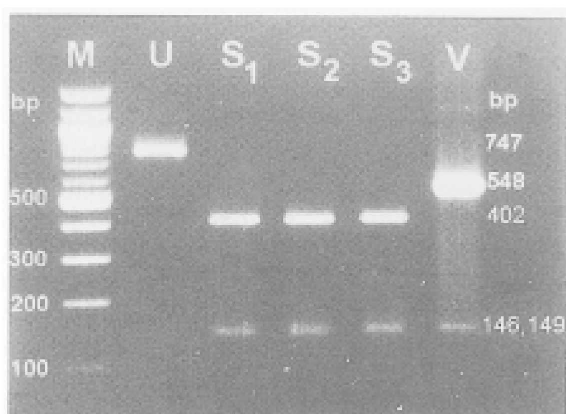

Fig 1. RFLP pattern of CPV digested with *Rsa* I. M: 100-bp marker; U: uncleaved PCR product (747 bp); S1-3: PCR product of samples 1-3 (CPV-2a or CPV-2b) digested by *Rsa* I gave of 149, 50, 146, and 402 bp; V: PCR product of vaccine strain (CPV-2) digested by *Rsa* I gave of 149, 50, and 548 bp.

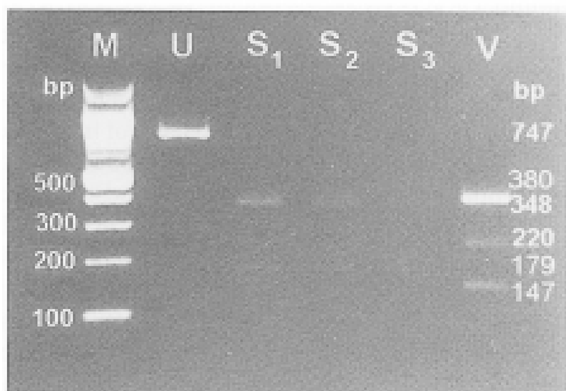

Fig 2. RFLP pattern of CPV digested with *Hph* I. M: 100-bp marker; U: uncleaved PCR product (747 bp); S1-3: PCR product of samples 1-3 (CPV-2a or CPV-2b) digested by *Hph* I gave of 220, 348, and 179 bp; V: PCR product of vaccine strain (CPV-2) digested by *Hph* I gave of 220, 147, and 380 bp.

Table 1. CPV-DNA detected in enteritic dogs of different age groups

Age	Number of samples	Number of CPV-DNA positive
1-2 months	8	5 (62.5%)
3-6 months	30	22 (73.3%)
>6 months	4	0 (0)
Unknown	13	7 (53.8%)

Discussion

Various methods have been applied aimed at detecting CPV infection in dogs with gastroenteritis. These include electron microscopy (EM) employing negative staining, virus isolation (VI) in cell/tissue

cultures, hemagglutination (HA) assays followed by HA inhibition (HI) by a CPV-specific antiserum, enzyme-linked immunosorbent assay (ELISA), and immunofluorescence.[25] The HA/HI assays have been routinely applied in numerous laboratories but non-specific agglutinin present in feces tends to interfere with these techniques. EM has proven a sensitive and reliable method for CPV diagnosis,[26] but is not practical for routine application. In the present study, we detected CPV DNA by semi-nested PCR. The percentage of enteric dogs testing CPV-positive was found to amount to 34/55 (61.8%). In 1995, Schunck et al detected CPV DNA from fecal specimens derived from enteritic dogs by single-round PCR. Using this method, they established the presence of CPV DNA in 54 out of 65 (83.1%) samples tested.[27] In 1993, Mochizuki et al detected CPV DNA from fecal samples of dogs with diarrhea by nested PCR. They found CPV DNA in 22 out of 59 (37.3%) fecal samples.[1] The above mentioned results indicate PCR to be useful as a routine diagnostic method for detection of CPV DNA in fecal specimens, particularly during the early and late stages of infection when the viral load might well be below the limit of detection by other, less sensitive methods. In the present study, we selected fecal specimens derived from clinically enteritic dogs. Applying semi-nested PCR, we found not all enteritic dogs tested to harbor CPV DNA. The causative agents of gastroenteritis in dogs may include parasites, bacteria or other viruses. A study conducted by Vieler and Herbst in 1995 has shown 17.2%, 12.4% and 2.5% of diarrhea occurring in dogs to be caused by parvovirus, coronavirus and other viruses (paramyxo-, picorna-, calici-, astro-, rota- and adenovirus), respectively.[28] In contrast, the healthy dogs used as controls were found negative for CPV DNA, indicating that CPV infection had not occurred in healthy dogs, hence, no virus was shed in their feces. Previous studies have demonstrated young dogs to be predisposed to develop CPV enteritis.[29,30] Our group's findings support this concept, in that CPV enteritis was exclusively found in non-vaccinated and/or very young (<6 months) dogs. More than 20 years have passed since CPV first was ascribed the potential to cause enteritis in dogs. Nowadays, most adult dogs are thought to be immune to CPV, mainly as a consequence of prior subclinical infection.

Since the tests currently employed to diagnose parvovirosis (HA/HI, ELISA, IF, etc.) lack the capacity required to differentiate between the vaccine strain CPV-2 and the wild strains CPV-2a and/or CPV-2b, employing PCR followed by RFLP may prove useful to achieve this end. In 1991, Parrish et al used a panel of monoclonal antibodies for antigen typing of CPV (CPV-2, CPV-2a, CPV-2b).[18] CPV typing at the genome level using site directed amplification may also represent a possible approach. An alternative approach is based on amplification of the VP2 gene followed by restriction enzyme mapping, thus permitting genotyping according to the restriction fragments created. Applying PCR and subsequent RFLP analysis with the restriction endonuclease *Rsa* I enabled us to differentiate CPV-2 from CPV-2a and CPV-2b.[31] In the present study, we employed both *Rsa* I and *Hph* I in order to distinguish CPV-2 from CPV-2a and CPV-2b. Upon investigating 34 CPV isolates, the results indicated that the vaccine strain CPV-2 did not cause CPV infection within the area examined. By applying these particular tests, we have not been able to differentiate between CPV-2a and CPV-2b. Further studies using DNA sequencing or competitive PCR using primers specific for the 3'-end of CPV may be required in order to distinguish between both wild strains.

In conclusion, PCR has proven distinctly advantageous for rapid and reliable diagnosis of CPV infection as shown by the presence of viral DNA in fecal specimens derived from enteritic dogs, thus providing a most essential tool for future control of viral spread and dissemination. In addition, differentiating the CPV-2 vaccine strains from both field strains CPV-2a and CPV-2b by restriction fragment length polymorphism analysis may prove advantageous in case CPV-2 dependent enteritis occurs among dogs immediately after CPV-2 vaccination. This might prove essential as a practical application to be drawn upon in order to counter claims implicating CPV-2 vaccines in disease onset shortly after vaccination. Moreover, PCR/RFLP can be applied to establish the cause of illness in dogs developing gastroenteritis despite or shortly after vaccination.

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