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GENOTYPING OF CANINE PARVOVIRUS BY PCR AND RFLP

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ABSTRACT
Canine parvovirus was first described in 1978, with the original isolate being termed CPV type 2 (CPV-2). Now recently since 2001, a new CPV mutant (CPV-2c) has been detected worldwide. The vaccines presently in use are CPV-2(a/b) strain thus there is a subsequent need to update current CPV vaccines. Hence, the objective of the present study was to detect and genotype CPV by polymerase chain reaction (PCR) and subsequent restriction fragment length polymorphism (RFLP) analysis of the PCR product. We investigated 20 fecal samples from vaccinated and unvaccinated dogs with signs of enteritis during January to May 2010 in private and Zaveri clinic located in Anand, Gujarat. The vaccine Nobivac Puppy DP was used as positive control. Out of the 20 specimens, fourteen samples from dogs with enteritis were found to be CPV DNA positive, using PCR assay. After establishing the difference between wild and vaccine strains using RFLP, we found that all virus strains in our study were either CPV-2b or CPV-2b variants, which differed from the vaccine strain. Molecular characterization and CPV typing are crucial in epidemiological studies for future prevention and control of the disease.

KEY WORDS: Enteritis, CPV mutant, Polymerase chain reaction, restriction fragment length polymorphism, restriction endonuclease, genotyping,

INTRODUCTION

Canine parvovirus (CPV) is classified into the family *Parvoviridae* (genus *Parvovirus*, subfamily *parvovirinae*). Canine parvovirus has a linear single-stranded (ss) DNA genome (5,323 nucleotides) with terminal palindromic structures. The genome encodes two structural (VP1 and VP2) and two non-structural proteins (NS1 and NS2). The non-structural proteins are translated from the open reading frame at the 3´end, while the other open reading frame at the 5´end encodes the structural proteins (Parrish *et al*., 1991).

CPV emerged in May 1978 worldwide (Parrish and Kawaoka, 2005) and was termed CPV type 2 (CPV-2) to distinguish it from CPV-1 (Minute virus of canines), which is antigenically different from CPV-2. CPV-2 emerged as a novel pathogen causing a new enteric and myocardial disease in dogs (Appel *et al.,* 1979). In 1979, CPV-2a was identified which differed from the original type 2 in five amino acid changes in the VP2 coat protein. The present evidence indicates that those amino acids are responsible for the antigenic and host-range viral properties (Truyen *et al*., 1995). Subsequently, CPV-2b was recognized among canine populations in 1984. The variant type 2b differs from type 2a in only two positions, Asn-426 to Asp and Ile-555 to Val (Parrish *et al*., 1991). Residue 426 is placed in a major antigenic site (epitope A) over the three-fold spike of the capsid and the mutation Asn-426 to Asp differentiates CPV-2b not only from CPV types 2 and 2a, but also from Feline parvovirus (FPV) and Mink enteritis virus (MEV). In 2001, a new CPV mutant Glu-426 produced by a Glutamate substitution at the same 426th residue was detected in Italy (Buonavoglia *et al.*, 2001). The Asp→Glu change at residue 426 of CPV-2c strain was due to a change (T→A) in the third codon position which created an *Mbo*II restriction site (GAGAA) unique to these strains.

Laboratory diagnosis of CPV is performed by demonstrating the presence of virus in feces. Employing PCR technique, primer-directed enzymatic amplification of specific DNA sequences can be accomplished 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. The objective of the present study was therefore to apply PCR to detect CPV DNA in fecal specimens derived from enteritic dogs. Furthermore, RFLP analysis was performed with the aim of differentiating between wild type and CPV-2 derived vaccine strains.

MATERIALS AND METHODS

The samples investigated consisted of 20 fecal specimens from vaccinated and unvaccinated dogs with signs of enteritis which were collected from the veterinary Zaveri clinic, Anand. The commercially available vaccine (Nobivac® Puppy DP, Intervet, lot no.SV-6-597) served as a positive control. The faecal samples were immersed in PBS to make 10 per cent (W/V) suspensions. The samples were then centrifuged at 3000 rpm for 20 min. at 4ºC and the clarified supernatants were preserved at -20ºC until further processed.

DNA extraction

DNA extraction from faecal samples was done by boiling method as per Schunck *et al.* (1995). The clarified faecal samples were centrifuged at 10,000 rpm at 4ºC for 15 min and the resulting supernatants were diluted 1:50 with sterile distilled water and boiled to 96ºC for 10 min. The same was used as DNA template for PCR assay.

Lyophilized vaccines (CPV-2) were resuspended in 1 ml of PBS and then processed using the same methodology.

PCR amplification

A 428 nt fragment of VP2 gene from position 4043 – 4470 was amplified following Pereira *et al.* (2000). The amplification consisted of an initial denaturation step at 94°C for 10 minutes, followed by 30 cycles comprised of a 30 second denaturation step at 94°C, a 2 minute annealing step at 55°C and a 2 minute extension step at 72°C, each. The amplification was concluded by a 10 minute elongation step at 72°C. Five 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 retrieved from Genbank with accession numbers M74849 and FJ349311 for CPV- 2b and CPV -2c, respectively. The CLUSTAL X program (NCBI) was used to compare the variant sequences of CPV-2b and CPV-2c from nt 4043 to 4470 and it was found that CPV-2b differs from CPV-2c by one nucleotide (nt 4064; T to A). The NEBcutterV2.0 *(http://tools.neb. com/NEBcutter2/index.php)*, was applied for selecting the restriction endonucleases most suitable for genotype specific cleavage. Accordingly, *Mbo*II, which recognizes GAGAA and cuts seven nucleotide ahead, was selected in order to distinguish CPV-2c from CPV-2b (Fig.1).

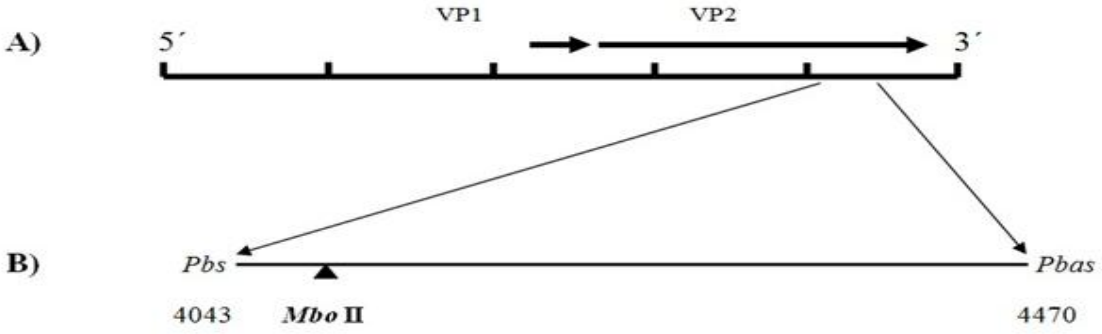

Fig.1 Genome structure and localization of VP1 and VP2 regions (A) amplified using the primers, *Pbs* and *Pbas*, and recognition sites of restriction enzyme (*Mbo* II) selected for RFLP analysis (B).

RESULT AND DISCUSSION

The product band of 427 base pairs (uncleaved PCR product) was visualized on a UV-transilluminator. In study, vaccine and 14 out of 20 samples from enteritic dogs proved positive for CPV DNA by PCR using CPV-2b specific primers, indicating that the field isolates were 2b strains. PCR using *Pabs/Pabas* were also used to detect CPV-2a strains in negative samples, but none were found positive. In order to differentiate between the wild strains, all specimens were subsequently subjected to RFLP analysis. Digestion by *Mbo*II produced a characteristic pattern distinguishing the vaccine strain from either wild strain. The characteristic product sizes of vaccine strain versus wild type obtained with enzyme are shown in Fig.2. About 11 out of 14 positive samples of dogs with enteritis were proved to harbour the wild strains CPV-2c, while vaccine and other three samples were CPV-2b.

Fig. 2 RFLP of *Pbs/Pbas* amplicons after digestion with *Mbo II* enzyme
M: Marker, UD: Undigested sample, V: Vaccine sample,
1-2: Undigested positive sample, 3-9: Digested positive sample

Various methods have been applied with the aim of detecting CPV infection in dogs with gastroenteritis such as electron microscopy (EM), virus isolation in cell/tissue cultures, hemagglutination (HA) assays followed by HA inhibition (HI) by a CPV-specific antiserum, enzyme-linked immunosorbent assay (ELISA), and immunofluorescence (Appel and Parrish, 1987). The HA/HI assays have been routinely applied in numerous laboratories but nonspecific agglutinin present in faeces tends to interfere with these techniques. EM has proven a sensitive and reliable method for CPV diagnosis (Mochizuki and Hida 1984), but is not practical for routine application. Mochizuki *et al.* (1993) detected CPV DNA from faecal samples of dogs with diarrhoea by nested PCR. In the present study, we detected CPV DNA by PCR and found CPV DNA in 14 out of 20 faecal samples. This indicates that PCR can be used as a routine diagnostic method for detection of CPV DNA in faecal 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.

Since the tests currently employed to diagnose parvoviruses (HA/HI, ELISA, IF, etc.) lack the capacity required to differentiate between the vaccine strain and the wild strains, employing PCR followed by RFLP may prove useful to achieve this end. Also, the PCR-based genotyping assay proposed by Pereira *et al.* (2000) failed to predict the antigenic type of field CPV-2 strains, as the strategy developed to amplify selectively type-2b CPVs was affected by the genetic variations that accumulated in CPV-2 genome. Antigenic analysis with monoclonal antibodies, sequence analysis of the VP2 or detection of SNPs by restriction enzyme analysis or by MGB assays are required to characterize the antigenic variants of CPV-2. Applying PCR and subsequent RFLP analysis with the restriction endonuclease *Mbo*II enabled us to differentiate CPV-2c from CPV-2b.

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. In addition, differentiating the vaccine strains from both field strains CPV-2b/2c by restriction fragment length polymorphism analysis may prove advantageous. It also appeared logical to opine that the present study revealed an important insight on genetic characterization of CPV. PCR-RFLP analysis of representative positive samples indicated a variant CPV-2c in the population under study, which demands for further investigation involving larger sample size and making comparative analysis with vaccine strains to deal with this important problem in young puppies.

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