

Detection and RFLP Analysis of Canine Parvovirus (CPV) DNA by Polymerase Chain Reaction (PCR) in a Dog

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Abstract: In this study, the detection of canine parvovirus (CPV) in a fecal sample from a dog with enteritis was performed for the first time using the polymerase chain reaction (PCR) in Turkey. The final PCR product was analyzed using the restriction fragment length polymorphism (RFLP) technique. RFLP analysis using Apa I and Eco RV restriction endonucleases revealed homology in the nucleotide sequence in at least the VP2 coding region of the virus DNAs detected in the fecal specimen and prepared from attenuated vaccine virus as a positive control.

Key Words: Canine parvovirus, diagnosis, PCR, RFLP

Bir Köpekte Canine Parvovirus DNA'sının Polimeraz Zincir Reaksiyonu (PZR) ile Tespiti ve RFLP Analizi

Özet: Bu çalışmada, Türkiye'de ilk kez enteritis bulgusu sergileyen bir köpekte parvovirus tespiti polimeraz zincir reaksiyonu (PZR) ile gerçekleştirildi. Elde edilen PZR ürünü restriksiyon endonükleaz enzimleri ile kesilerek RFLP analizine tabi tutuldu. Apa I ve Eco RV enzimleri ile yapılan RFLP analizinde, dışkıda tespit edilen ve pozitif kontrol olarak canlı attenüe parvovirus aşısından hazırlanan DNA'ların en azından VP2 kodlayan bölgelerinde homoloji tespit edildi.

Anahtar Sözcükler: Canine parvovirus, tanı, PZR, RFLP

Introduction

Canine parvovirus (CPV) is an important agent causing severe enteritis and systemic disease in dogs throughout the world. CPV is an autonomously replicating parvovirus, and is genetically related to the feline panleukopenia virus (FPLV), mink enteritis virus (MEV) and blue fox parvovirus (BFPV). These viruses are accepted as host range variants of FPLV belonging to the genus *Parvovirus* in the family *Parvoviridae* (1). Parvoviruses contain a linear ssDNA genome of about 5.3 kb. The genome has two open reading frames (ORF). The first ORF encodes at least one nonstructural protein (NS1), and the second ORF encodes two capsid proteins (VP1 and VP2) which are translated from alternatively spliced mRNA (2).

The main source of the infection seems to be the feces of infected dogs because more than 10^9 virus particles per gram of feces can be shed during the acute phase of the enteric disease. Therefore, feces are accepted as a suitable material to detect the virus in the enteric form of the disease (3). In previous researches (4,5), it was shown that the PCR technique is a rapid, sensitive and specific technique for CPV detection in contaminated fecal samples compared to virus isolation and hemagglutination assay. In addition, Mochizuki et al. (4) reported that this technique can be applied to a spoiled sample in which viruses have been inactivated. In this paper, the first detection and subsequent preliminary genome characterization of CPV by PCR in a dog with enteritis are reported.

Case Definition

A six-month-old bitch suffering from mild enteritis was submitted to the internal clinic of the Veterinary School, Yüzüncü Yıl University, Van, Turkey. The dog was a member of a colony of 10 or more individuals and it was detected from the anamnesis that she had not received a series vaccine against critical diseases. The dog responded to the therapy given and recovered to full health 10 days after the onset of the disease.

During the diarrhoeic phase, a fecal specimen was collected by rectal swabbing in order to detect DNA of CPV. A swab sample was immediately immersed into 500 µl of TE buffer (10 mM Tris-HCl, pH 7.8; 1 mM EDTA) and transported to the laboratory. CPV DNA was extracted using a Phenol/Choloroform/Isoamylalchol (25:24:1, v/v/v) mixture in an equal volume (400 µl) of the sample used. DNA extraction was repeated at least twice until the interphase was completely cleared after brief spinning at 6000 rpm for 5 min. The DNA was then precipitated by adding an equal volume of isopropanol in the presence of a 1/10 volume of 3 M Na-Acetate (pH 5.2) at -80 °C for 1 h. The total DNA was isolated by centrifugation at 12,000 rpm for 10 min, which was followed by washing the pellet with 70% ethanol and subsequent drying at 37 °C. The DNA was dissolved in 20 µl of sterile distilled water and used for PCR amplification. For this purpose, the complete VP1-VP2 coding genome region of CPV DNA was targeted (Figure 1). The primer pair designed for PCR amplification is given in the Table. The PCR amplification was performed in a total of 30 µl by adding 3 µl of DNA extracted to the PCR master mix containing 75 mM Tris-

HCl (pH 8.8), 20 mM NH₄(SO₄)₂, 1.5 mM of MgCl₂, 10 pmole of each primer, 0.2 mM of dNTP, and 0.5 U of Taq DNA polymerase (MBI, Fermentas, Lithuania) in a final volume of 30 µl. The thermal cycler (Techne, Oxford, UK) program was set up as follows: an initial denaturation step at 94 °C for 6 min was followed by a cycle of 60 s at 52 °C, 150 s at 72 °C and 60 s at 94 °C, repeated 40 times. Amplification was terminated by a final extension at 70 °C for 10 min. At the end of the reaction, it was expected to amplify a 2245 bp DNA product. During the synthetic amplification of the VP1-VP2 coding region of CPV, attenuated live CPV vaccine virus (Parvoid 2, Solvay Anim. Health, Inc., MN, USA) was used as a standard positive control. The resulting DNA products (amplicons) were separated on 1.5% agarose gels containing 25 µg ethidium bromide after electrophoresis at 80 V for 30 min. The DNA bands were observed under UV light and photographic records were made. For RFLP analysis, the PCR products were digested using Apa I and EcoRV at 37 °C for 1 h. Samples were then analyzed on 1.7% agarose gels to determine the cleavage patterns of the amplicons (Figure 2).

Results and Discussion

The PCR application revealed an intensive product in the fecal sample in parallel with the positive control DNA prepared from the attenuated live CPV vaccine. The product was 2245 bp in length, as expected. RFLP analysis of both PCR amplicons obtained from the fecal sample and control DNA revealed the same digestion pattern by means of cleavage with Apa I and Eco RV

Primer	Sequence (5'→3')	Position at CPV [¶]
CPV-P1 (Forward)	ATggCACCTCCggCAAAGa	2285-2303
CPV-P2 (Reverse)	TTTCTAggTgCTAgTTgAg	4512-4530

Table. Sequences and positions of the primers used in the study.

[¶] The sequence was retrieved from NCBI, USA (Accession Number NC_001539).

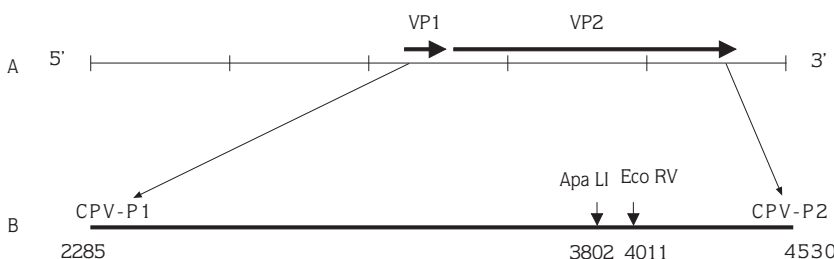


Figure 1. Genome structure and localization of VP1 and VP2 regions (A) amplified using the primers, CPV-P1 and CPV-P2, and recognition sites of restriction enzymes selected for RFLP analysis (B).

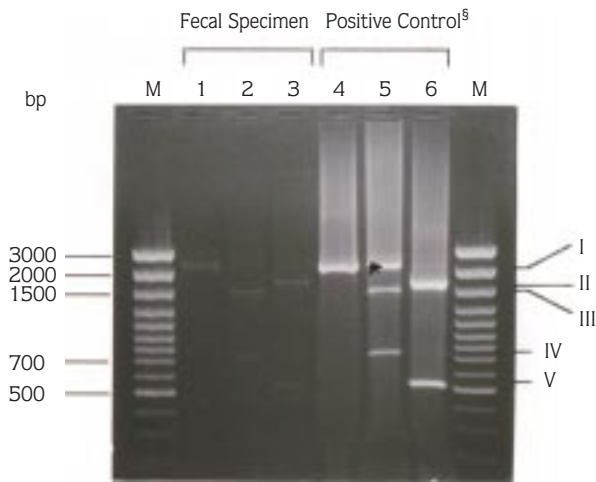


Figure 2. Restriction patterns of the VP2 gene of canine parvovirus after digestion with Apa LI and Eco RV.

Lane 1, PCR product from fecal sample; Lane 2, Apa LI digest; Lane 3, Eco RV digest; Lane 4, PCR product from control DNA; Lane 5, Apa LI digest; Lane 6, Eco RV digest; and M, 100 bp DNA ladder (MBI, Fermentas, Lithuania). Roman numerals on the right side of the figure indicate the sizes of PCR products and restriction fragments created by the enzymes: I, 2245 bp; II, 1726 bp; III, 1517 bp; and IV, 728 bp; V, 519 bp. § Positive control was prepared from live attenuated vaccine virus (Parvovoid 2, Solvay Anim. Health, Inc., MN, USA). * Partial digestion with Apa LI of PCR product yielded from control DNA.

restriction enzymes. Digestion with Apa LI created 728 bp and 1517 bp bands while digestion with Eco RV produced bands of the same number but of the different sizes (519 bp and 1726 bp), as expected from the known sequence of CPV (NCBI Accession Nr; NC_001539).

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Meanwhile, partial digestion was detected in the cleavage of control DNA with Apa LI, as shown by an asterisk on Figure 2, probably caused by a high copy number of the DNA product.

In conclusion, the DNA of CPV was detected in a diarrhoeic dog using PCR technology for the first time in Turkey. This result indicates that for the direct diagnosis of CPV infection PCR can be used as a reliable and fast technique in comparison to cultural isolation or hemagglutination assay, as pointed out before by many authors (4,5). An additional advantage of this technique is that the product obtained from PCR can be further analyzed by means of various genetically important techniques, such as RFLP, in order to obtain valuable information on the structural properties of viral genomes amplified from various origins (6). As we aimed to amplify the second ORF, in which large structural polyprotein is encoded, it was expected that the results from RFLP analysis might have revealed information on the putative antigenic diversities on the viral surface between field and vaccine viruses. Therefore, RFLP analysis was performed on the VP2 coding region of CPV DNA, which is an important capsid protein, using Apa LI and Eco RV. Surprisingly, RFLP revealed the same cleavage patterns with respect to the restriction enzymes used, which indicates obvious nucleotide homology in the concerned region of the genome of the viruses (Figure 2). Based on this and a previous report (7), we will carry out further investigations on CPV in order to understand the possibilities of the occurrence of mild infection caused by attenuated vaccine strains in mixed populations.