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Phylogenetic analysis of canine parvovirus isolates from Mathura, India

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ABSTRACT

Canine parvovirus (CPV) is an important pathogen causing acute hemorrhagic gastroenteritis in dogs and myocarditis in pups. The present study deals with an analysis of partial nucleotide sequences of the VP1/VP2 gene of CPV isolates from Mathura, India to establish their phylogenetic relationship with other CPV isolates. Out of 100 samples from dogs showing the clinical signs of gastroenteritis viz., vomiting, diarrhea and dysentery, 63 were found positive for CPV-2 by polymerase chain reaction (PCR). Among the 63 positive samples, eight samples were processed further for nucleotide sequencing. Phylogenetic analysis revealed that the CPV variants were not only closely related among themselves but also showed minimum divergence from their ancestors, such as MEV, indicating very little divergence since their origin. From the study, it may be concluded that canine parvovirus-2 variants may represent a potential threat to canine populations. Thus more efforts is required to increase epidemiological monitoring and surveillance, along with the measures necessary to control this disease in the canine population, and to assess the efficacy of the current vaccines.

Key words: canine parvovirus, dog, molecular characterization, PCR, phylogenetic analysis

Introduction

Canine parvovirus (CPV) was first identified in 1978 (SINGH et al., 2013). Since then it has spread very quickly and has become endemic in most parts of the world (GUY, 1986).

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The virus has a genome consisting of single stranded DNA of 5.2 kbp length. It causes a highly contagious disease that can spread rapidly through a population of dogs with high morbidity (100%) and frequent mortality up to 10% (APPEL et al., 1978; SINGH et al., 2013). The virus has two different strains i.e. CPV-1 and CPV-2. With a high evolution rate, three CPV-2 variations have emerged since its first isolation (SHACKELTON et al., 2005). CPV-2 variations emerged in 1980 in the United States (PARRISH et al., 1988). CPV-2b replaced CPV-2a in many regions of the US after 1986 (PARRISH et al., 1991). In 2001, CPV-2c was first reported in Italy and then in some other countries, including the USA and Brazil (HONG et al., 2007; PERVEZ et al., 2007), Vietnam (NAKAMURA et al., 2003) and India (NANDI et al., 2010). In India, CPV-2 was first reported in 1982 by Ramadass and Khader and since then has been a major concern of animal health professionals. To date a large number of outbreaks have been reported from different parts of India in dogs involving different variants of CPV viz., 2a, 2b and 2c (CHINEHKAR et al., 2006; BISWAS et al., 2006; NANDI et al., 2009a and 2009b). The Polymerase Chain Reaction (PCR) technique has been widely applied for early and confirmatory laboratory diagnosis of the disease, due to its high sensitivity and specificity (SCHUNCK et al., 1995; NANDI et al., 2006, 2008 and 2010; SINGH et al., 2013).

However, scanty literature is available on the antigenic and genetic characterization of CPV isolates from India. Thus, the present study was envisaged to genetically characterize CPV isolates from Mathura, India and to compare them with other published CPV strains.

Materials and methods

Study design, area, fecal sample preparation. During the present study, a total of 100 fecal samples were collected from dogs, presented to the Teaching Veterinary Clinical Complex, DUVASU, Mathura, India and showing clinical signs of gastroenteritis. The fecal samples were collected in the form of a rectal swab, using pre-sterilized swabs, and immediately transferred in a ratio of 1:9 to labeled sterile vials with Hank's balanced salt solution (HBBS) containing streptomycin (100 mg/L) and penicillin (1 Lakh IU/L). The swabs were properly rinsed in the HBSS vials and taken out. The remaining fecal contents in the HBSS were centrifuged at 10,000 rpm at 4 °C for 3 min in a refrigerated centrifuge. The supernatants were pipetted out and filtered through a disposable syringe filter (0.45 μm). Inactivated canine nasal parvo vaccine, supplied by Indian Immunological Limited, Hyderabad, was used as a positive control of CPV and a stool sample from a healthy dog, processed in the same way, was used as a negative control.

Genomic DNA extraction and PCR amplification of CPV-2. The genomic DNA from the fecal samples was extracted by the phenol chloroform method (SAMBROOK and RUSSEL, 2001). To remove inhibitory substances, 200 μL of sample was treated with sodium dodecyl sulphate (SDS) and proteinase K, with a final concentration of 1% and 250 μg/ ml, and kept at 56 °C for 30 minutes in a water bath. Then, 200 μL of Tris saturated phenol, chloroform and isoamyl alcohol (25:24:1) was added to 200 μL of the

sample in an Eppendorf tube and mixed thoroughly. It was centrifuged at 10,000 rpm for 5 min at 4 ºC and the supernatant was collected in another Eppendorf tube. Then 1/10th volume of 3M sodium acetate (pH 5.5) and 1 mL of chilled ethanol was added, mixed and kept at -20 ºC overnight. After that the tubes were centrifuged at 12,000 rpm for 15 min at 4 ºC. The supernatant was discarded and pellets washed with 500 µL of 70% ethanol, followed by centrifugation at 12,000 rpm for 2 min. The ethanol was discarded and the pellets dried at 37 °C and re-suspended in 20 µL of nuclease free water. The purity of DNA was checked by UV-spectrophotometer by taking 260/280 nm ratio, which was found to be between 1.7 to 1.8 and the concentration of DNA was 20 ng/µl.

The set of primer {pCPV forward primer-5' GAA GAG TGG TTG TAA ATA ATT-3' (21 mer) and pCPV reverse primer-5'-CCT ATA TCA CCA AAG TTA GTA G-3' (22mer)}, used to amplify the partial VP1/VP2 gene of the CPV in PCR, was custom designed to yield an amplicon of 681bp (PEREIRA et al., 2000). PCR was performed in 0.2 mL PCR tubes (Eppendorf) with a reaction volume of 50 µL. The reaction mixture contained 200 µM dNTPs, 10 pmol of each primer, 5 µL of 10x Taq DNA polymerase buffer containing 15 mM $MgCl_2$, 5 µL of extracted DNA as template and 1 µL of Hotstar DNA polymerase (1 IU/ µL) and nuclease free water to make the volume up to 50 µL. The cycling conditions comprised: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, primer annealing at 55 °C for 2 min, and extension at 72 °C for 30 sec. Then, the final extension was done at 72 °C for 10 min. After completion of PCR, the amplified products were electrophoresed on 1% agarose gel, stained with ethidium bromide and then visualized under the UV transilluminator. The PCR products were purified from the gel using a gel extraction kit (Genei Pvt Ltd., Banglore) as per the manufacturer's protocol, and then sequenced.

Phylogenetic analysis. For phylogenetic analysis of these recent isolates with the rest of the previous isolates included in the tree construction, the sequences were retrieved from the GenBank. Molecular sequences (nucleotide and amino acids) were aligned against the other published CPV VP1/VP2 gene sequences using the clustal W/X algorithm (THOMPSON et al., 1994 and 1997), implemented in the OMIGA 2.0 program (Oxford Molecular Ltd, UK) and refined in clustal X (THOMPSON et al., 1997). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (TAMURA et al., 2007). The TAMURA and NEI (1993) model of nucleotide substitution with gamma-distribution of among site rate heterogeneity (with 8 categories) (termed as TrN + G model) available in MEGA was used to construct the trees. The tree topologies were evaluated using 10,000 replicates of the data set. The tree thus generated was produced in Lotus Freelance Graphics 97.

Results

In the present study, a total of 100 samples were tested, out of which an amplicon of 681bp was obtained in 63 samples showing the presence of canine parvovirus infection in these dogs. The positive control also showed similar amplification of DNA, while in the negative control no such amplification was visualized on an agarose gel. Nucleotide sequences were aligned, phylogenetically characterized and compared with various previous isolates CPV-2 isolates, mink enteritis virus-1 and 2 (MEV-1 and 2) available on the NCBI and CPV-2 vaccine, with the help of MegAlign software. In the Phylogenetic tree (Fig. 1), all the field isolates isolated in the present study were grouped in one group, along with isolates from China, Brazil, Bareilly, Kerala-2 and the vaccine strain indicating the similarity between them. However, the isolates (Pondicherry 1 and 2; Kerala-1) could be distributed in another genetic group.

The nucleotide (nt) divergence among the field isolates (Mathura 1-8) sequenced in this study and vaccine strains and other representative isolates (MEV-1, CPV-2a, CPV-2b Pondichery, CPV-2b China, CPV-2b Bareilly, CPV-2b Brazil, CPV-2b Iyrland, CPV-2b Pondichery-2, CPV-2b Kerala) is shown in Table 1. From the table it can be seen that the nucleotide divergence between Mathura isolates and CPV-2b Pondichery-2 isolates is higher (5.8%) compared to that among the recent field isolates from Mathura (0.00%). Alignment of partial nucleotide sequences of the VP1/VP2 region revealed common substitution in the recent field isolates, in comparison with MEV. These differences were found at various synonymous positions (nt 2 T—A, 12 A—T, 42 A—C, 99 T—C, 108 A—G, 555 A—C, 591 T—C, 623 A—G, 635 C—G, 654 A—G) compared to the MEV.

Table 1. Percentage divergence of partial nucleotide sequences of VP1/VP2 gene of CPV isolates

		1	2	3	4	5	6	7	8	9	10	11
MEV 1	1											
CPV 2a	2	0.9										
CPV 2b Pondicherry	3	3.7	2.8									
CPV 2b China	4	0.9	1.8	2.8								
CPV 2b Vaccine	5	0.9	1.8	2.8	0.00							
CPV 2b Bareilly	6	0.9	1.8	2.8	0.00	0.00						
CPV 2b Mathura	7	0.9	1.8	2.8	0.00	0.00	0.00					
CPV-2b Brazil	8	0.9	1.8	2.8	0.00	0.00	0.00	0.00				
CPV 2b Ireland	9	1.9	2.8	3.8	0.9	0.9	0.9	0.9	0.9			
CPV 2b Pondichery2	10	6.9	5.8	6.9	5.8	5.8	5.8	5.8	5.8	6.9		
CPV 2b Kerala	11	1.9	2.8	3.8	0.9	0.9	0.9	0.9	0.9	1.9	4.8	

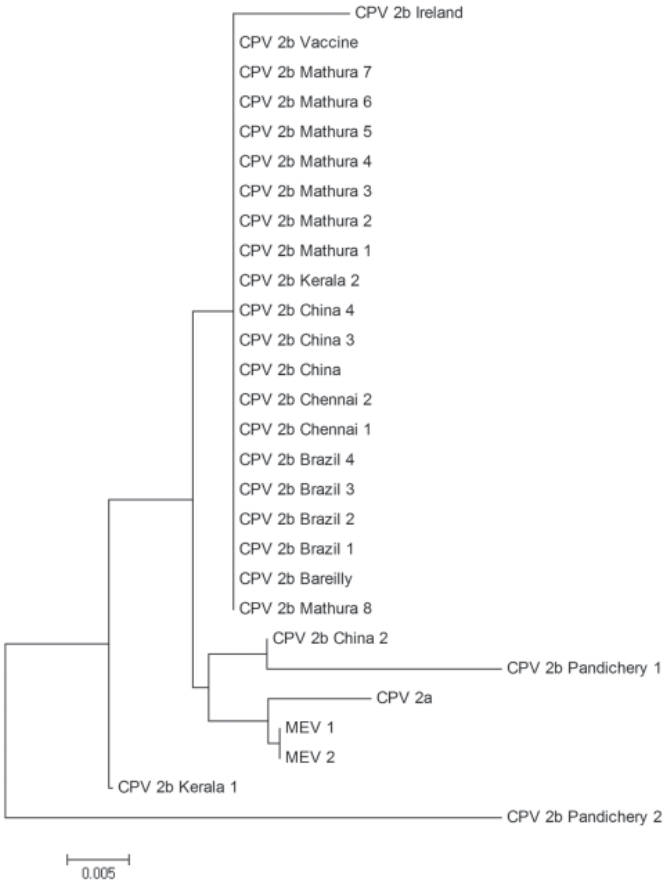

Fig. 1. Neighbor joining tree constructed from VP1/VP2 gene sequences showing the phylogenetic relationships between CPV isolates. (Horizontal branch lengths are drawn to scale)

Discussion

Phylogenetic analysis also revealed that CPV variants are not only closely related among themselves, but also show minimum divergence from their ancestors, such as MEV, indicating very little divergence since it was first identified in 1978 (APPEL et al., 1978). There was no appreciable variation within the various isolates. The variations that were observed nevertheless played a crucial role in the evolution of different variants in several geographical regions of the world. NANDI et al. (2009a) also reported 1.7% divergence of IVRI CPV-2b isolates from the Brazil CPV-2a isolate. They further reported

that IVRI isolates closely resembled an Italian strain. Various researchers (PARRISH et al., 1991; STEINEL et al., 1998; NANDI et al., 2009a and 2009b) reported that CPV-2a and CPV-2b co-existed in different ratios in various countries, indicating no evolutionary advantage of one type over another, and this co-existence has not evolved under immuno-selective pressure from vaccines. However, regular monitoring, surveillance and full genome sequence analysis will further help to discover the exact places of mutations, and will provide the possible regions of antigenic variation in canine parvovirus.

From the study, it may be concluded that canine parvovirus-2 variants may represent a potential threat to canine populations, thus warranting efforts to increase epidemiological monitoring and surveillance, along with the necessary measures to control this disease in canine population and to assess the efficacy of the current vaccines.

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SAŽETAK

Pasji parvovirus važan je uzročnik hemoragijskog gastroenteritisa u odraslih pasa i miokarditisa u štenadi. U ovom su istraživanju djelomično analizirane nukleotidne sekvencije gena VP1/VP2 izolata pasjeg parvovirusa iz Mathure u Indiji sa svrhom da se njihova filogenetska svojstva usporede s drugim izolatima toga virusa. Od 100 uzoraka izdvojenih iz pasa s gastroenteritisom odnosno s povraćanjem i proljevom, 63 su bila pozitivna na pasji parvovirus 2 upotrebom lančane reakcije polimerazom. Od toga je osam uzoraka uzeto za određivanje njihova nukleotidnog slijeda. Filogenetska analiza je pokazala da varijante pasjeg parvovirusa nisu bile samo međusobno usko srodne, već su s neznatnim skretanjem bile srodne i sa svojim predcima, kao što je virus enteritisa američke vidrice, što upućuje na njihove neznatne razlike od njihova nastanka. Može se zaključiti da varijante pasjeg parvovirusa 2 predstavljaju moguću prijetnju za populaciju pasa. Potrebno je uložiti više napora u smjeru epizootioloških istraživanja i donošenja mjera nadzora te kontrole ove zarazne bolesti zajedno s naporima za procjenu učinkovitosti postojećih cjepiva.

Ključne riječi: pasji parvovirus, pas, molekularna obilježja, lančana reakcija polimerazom, filogenetska analiza