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Short communication

Direct typing of *Canine parvovirus* (CPV) from infected dog faeces by rapid mini sequencing technique

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ABSTRACT

Canine parvovirus (CPV) is a non-enveloped single stranded DNA virus with an icosahedral capsid. Mini-sequencing based CPV typing was developed earlier to detect and differentiate all the CPV types and FPV in a single reaction. This technique was further evaluated in the present study by performing the mini-sequencing directly from fecal samples which avoided tedious virus isolation steps by cell culture system. Fecal swab samples were collected from 84 dogs with enteritis symptoms, suggestive of parvoviral infection from different locations across India. Seventy six of these samples were positive by PCR; the subsequent mini-sequencing reaction typed 74 of them as type 2a virus, and 2 samples as type 2b. Additionally, 25 of the positive samples were typed by cycle sequencing of PCR products. Direct CPV typing from fecal samples using mini-sequencing showed 100% correlation with CPV typing by cycle sequencing. Moreover, CPV typing was achieved by mini-sequencing even with faintly positive PCR amplicons which was not possible by cycle sequencing. Therefore, the mini-sequencing technique is recommended for regular epidemiological follow up of CPV types, since the technique is rapid, highly sensitive and high capacity method for CPV typing.

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1. Introduction

Canine parvoviral enteritis is one of the fatal diseases of dogs and many other wild carnivores. The disease has worldwide prevalence (Appel et al., 1979; Burtonboy et al., 1979). Canine parvoviral enteritis had emerged during 1970s as a new infectious disease of pups characterized by either gastro-enteritis or myocarditis and the etiological agent was named as *Canine parvovirus* (CPV) type 2 (Appel et al., 1979; Burtonboy et al., 1979). CPV is a single stranded, non-enveloped DNA virus with icosahedral capsid. The CPV type 2 virus underwent genetic and antigenic changes to become CPV type 2a, subsequently to type 2b and later as type 2c (Buonavoglia et al., 2001; Nakamura et al., 2004; Parrish et al., 1991). Globally, original CPV type 2 virus was replaced with the variant types (2a–2c) in the outbreaks.Live modified vaccines are being used widely to control the disease. In spite of the widespread use of vaccines, the disease remains to be one of the major problems of pets (Chinchkar et al., 2006). Though, the interference from maternal antibodies is considered as primary reason for vaccine failure, antigenic variation between the current vaccine strains (CPV 2) and the prevalent virus types was also indicated as a possible reason for vaccine failure albeit with conflicting reports (Chinchkar et al., 2014; Decaro et al., 2008; Spibey et al., 2008).

Antigenic variants of CPV 2 differ from the original type 2 virus at 4–5 amino acid residues of the capsid protein VP2. However, the amino acid variation among the antigenic variants (2a–2c) is restricted to one amino acid within the VP2 protein and the variation was due to single nucleotide polymorphisms (SNPs) at the DNA sequence level. Restriction enzyme digestion of short PCR fragments of CPV genome was used for the discrimination of CPV-2c from other variant strains (Buonavoglia et al., 2001). A series of realtime PCR based minor groove binder (MGB) assays were performed for detecting the CPV type specific SNPs and the drawback

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of this technique is that several individual MGB assays are needed to differentiate types 2, 2a–2c in a sample (Decaro et al., 2006a,b).

Single nucleotide extension using the mini-sequencing technique is one of the available methods to identify SNPs (Sobrino et al., 2005). A mini-sequencing based typing technique was tested successfully to type CPV 2, the CPV 2 variants and FPV (Naidu et al., 2012). This technique was earlier assessed using laboratory isolates of CPV (Naidu et al., 2012). Tedious virus isolation and subsequent typing do not provide much advantage for the mini-sequencing technique over the cycle sequencing. Therefore, in the present study, utility of this technique in determining the CPV types directly from the CPV suspected field samples was evaluated and the results were compared with typing by standard cycle sequencing.

2. Sample collection

Fecal swabs were collected from dogs showing typical signs of parvoviral enteritis such as vomiting and bloody diarrhoea. The samples were collected from various veterinary hospitals located in four different provinces of India. The provinces were two western states of Gujarat and Maharashtra; two southern states of Tamil Nadu and Andhra Pradesh. A total of 84 fecal swabs were collected and processed for CPV genome identification by PCR. Few of the samples were also passaged in A72 cells for virus isolation.

3. Sample processing and PCR

The swab samples were stored and transported in sterile 0.5 ml minimum essential medium (MEM) with 2x antibiotics and antimycotic solution (100x antibiotic and antimycotic solution, Sigma Aldrich, USA; 10,000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per ml). The swab materials were subjected to vortexing and the swab contents were squeezed along the sides of tubes. The samples were made up to 1 ml using MEM and centrifuged at 6000*g* for 10 min. The supernatant was passed through 0.2 μ filter and used for PCR or virus isolation. DNA extraction was performed from 300 μl of the sample using DNAzol® reagent (Life technologies, USA) as per manufacturer's instructions.

4. PCR using CPV Ext For and CPV Ext Rev primers

The DNA samples were subjected to PCR using CPV Ext For (5′-GGCAAACAAATAGAGCATTGG-3′) and CPV Ext Rev (5′CCCAAATTTGACCATTTGGAT-3′) primers as described by Naidu et al. (2012). Out of 84 fecal samples 76 were positive in PCR using CPV Ext for/rev primers. All the PCR negative samples (n = 8) were passaged 5 times in A72 cells. The 5th passage samples were again subjected to PCR. All these samples turned PCR negative even after five blind passages in A72 cells, confirming that these samples were CPV isolation negative.

All the 76 PCR positive samples were analysed by mini-sequencing to determine the CPV types and the mini-sequencing was performed using un-purified PCR products.

5. CPV typing by mini-sequencing

Mini-sequencing reaction was performed as per the method described by Naidu et al. (2012). Briefly, the unpurified PCR products were treated with 5 units of Exonuclease 1 (Exo1, New England Biolabs, USA) and 3 units of shrimp alkaline phosphatase (SAP, New England Biolabs, USA) to remove/dephosphorylate the unutilised primers and dNTPs. Exo1 and SAP were inactivated by incubating at 75 °C for 15 min. The Exo/SAP treated template was directly utilized for mini-sequencing reaction. Five primers were used for the multiplex mini-sequencing reaction (Naidu et al., 2012). These primers were one base short of the selected SNPs in CPV genome (3685, 3699, 3753, 4062 and 4064 of the CPV genome; M38245). In the mini-sequencing reaction, the primers were extended one base by one of the four fluorescently labelled ddNTPs. The incorporated ddNTPs reflected the SNPs of CPV genome. After the mini-sequencing reaction, 1 unit of SAP was added and the mix was incubated at 37 °C for 1 h to remove 5' phosphoryl groups followed by SAP inactivation at 75 °C for 15 min. Length of single base extended primers was analysed in a 36 cm capillary using POP-7 polymer matrix (Applied Biosystems, USA) in an ABI PRISM 3130xl genetic analyser (Applied Biosystems, USA).

Data was collected using the 3130xl (version 3) software and analysis was done using Gene-mapper v.3.7 software (Applied Biosystems, Foster City, USA). Peaks with relative fluorescence intensity (RFU) higher than 100 were considered positive. SNP positions were identified by the primer length and a mini-sequencing signature was developed as described earlier by Naidu et al. (2012).

CPV types were identified from all the 76 PCR positive samples by mini-sequencing reaction. The mini-sequencing results showed that 74 samples belonged to CPV type 2a and the remaining two samples were CPV type 2b.

6. CPV typing by cycle sequencing

Twenty three of the randomly selected CPV type 2a samples and both the CPV type 2b samples were subjected to cycle sequencing also. All the twenty five PCR products were gel purified as per manufacturer's instructions (Qiagen, Germany) and subjected to cycle sequencing. Gel purification of the PCR products was performed by pooling multiple PCR reactions from a sample, if required. Quality of cycle sequencing result was poor for three of the samples, which produced faint bands in PCR (Fig. 1). Therefore, these samples were passaged 5 times in A72 cells. Intense bands in PCR could be obtained from the 5th passage sample. These PCR products were purified and subjected to cycle sequencing. Typing results of cycle sequencing matched with the mini-sequencing results for all the 25 samples (Table 1).

7. Discussion

Host switching and host adaptation were significant phenomena in FPV and CPV. CPV type 2 evolved by successful canine host adaptation of FPV or a FPV like feline virus. The original CPV type 2 was predominantly a canine virus. The virus further evolved as CPV type 2a by expanding its host range to include felines also. Though CPV is a DNA virus, the evolution rate of the virus is similar to some of the rapidly evolving RNA viruses (Shackelton et al., 2005). The overall dN/dS ratio of the VP2 gene was low indicating purifying selection. However, few amino acid residues within VP2 are under positive selection, which provides adaptive potential for the virus (Hoelzer et al., 2008). Amino acid 300 and surrounding residues of VP2 protein are under such positive selection. These residues are surface exposed, involved in host cell receptor (Transferrin Receptor – TfR) binding and probably playing a role in host adaptation. Similarly, residue 426 of VP2 protein is also under very high positive selection and suspected to have a role in immune evasion or viral pathogenesis (Hoelzer et al., 2008; Nakamura et al., 2004). Therefore, it is very important to analyse the prevailing CPV types to track the virus evolution and for subsequent implementation of control measures. Mini-sequencing technique is a highly reliable and rapid method for typing CPV from very high number of field samples. Mini-sequencing reaction has multiplexing capability upto 10 primers. Therefore, the newer types and additional mutations can also be added in future.

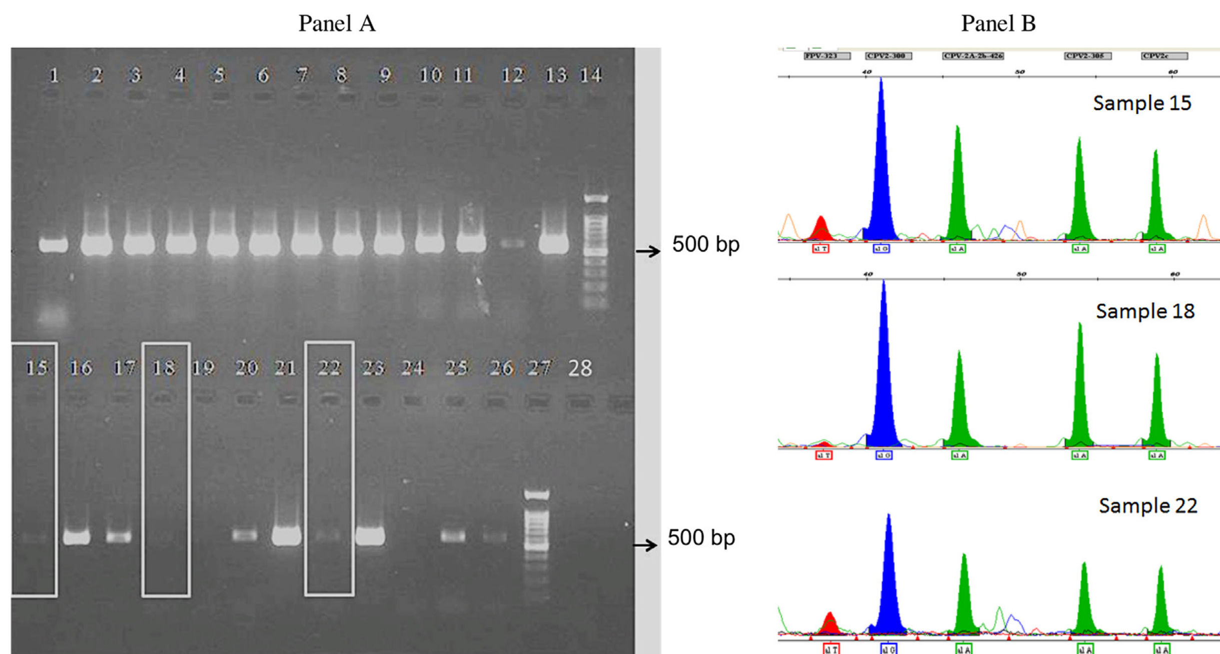

Fig. 1. Fecal samples were subjected to PCR using Ext for and Ext Rev primers (panel A). PCR amplicon of 576 bp was observed for the positive samples. Samples in lane No 19 and 24 were declared negative. Lane 14 and 27 represent the 100 bp DNA ladder. Lane 28 represents negative control. Faint bands were observed in lane numbers 15, 18 and 22. Typing by mini-sequencing was successful from these samples (Panel B) whereas the cycle sequencing did not yield readable peaks. CPV types were determined by mini-sequencing without any ambiguity from these samples in spite of the visibly low template quantity. All the three samples were typed as CPV type 2a.

Table 1
CPV typing by mini-sequencing: All the PCR positive samples were typed by mini-sequencing reaction. Additionally, 25 of the positive samples were typed by cycle sequencing of PCR products. The typing results of mini-sequencing matched 100% with typing by cycle sequencing. *20 of these samples were typed by cycle sequencing also; #all these samples were typed by cycle sequencing also.

S. No.	Province/Location		Number of Samples tested	Positive by PCR	Mini-sequencing result	
					CPV type 2a	CPV type 2b
1.	Gujarat		3	3	3	0
2.	Maharashtra		7	6	6	0
3.	Tamil Nadu	Chennai	45	40	39*	1#
		Other parts of Tamil Nadu	25	23	23	0
4.	Andhra Pradesh		4	4	3#	1#
Total			84	76	74	2

Antigenic variation among the prevailing types of CPV are based on one amino acid variation within VP2 protein or in turn on one nucleotide difference at DNA level. Therefore, CPV types can only be identified by SNP detection methods (Chinchkar et al., 2006; Martella et al., 2006). CPV typing by haemagglutination inhibition assay using type specific monoclonal antibodies had been used. Sensitivity of the assay is less; availability of type specific monoclonal antibodies is a major limiting factor for using the assay. The type specific SNPs were detected by MGB based real time PCR; but the costly assay has to be done in several individual reactions for differentiating the CPV types (Decaro et al., 2006a,b, 2008). Cycle sequencing is practical only on representative samples and sensitivity of cycle sequencing is much lesser than mini-sequencing.

Utility of mini-sequencing reaction for typing CPV was demonstrated earlier, using laboratory isolates (Naidu et al., 2012). However, virus isolation followed by mini-sequencing is time consuming and the standard cycle-sequencing is the preferred choice in such a case. In the present study, the assay was performed directly using CPV suspected dog fecal samples. CPV types were determined unambiguously from these samples and the mini-sequencing results matched 100% with typing by cycle sequencing.

As per the earlier report, dog feces containing $\leq 10^1$ FAID50 virus or <100 copies of templates were typed by mini-sequencing method, whereas, the regular cycle sequencing required $>10^3$ $FAID_{50}$ of virus in recording readable peaks and detecting the CPV types with certainty (Naidu et al., 2012). During the present study also, cycle sequencing results were not in readable quality for some of the samples (n = 3), which produced faint PCR amplicons (Fig. 1). However, mini-sequencing technique identified the CPV types of these samples without any ambiguity (Fig. 1). All the three samples were typed as CPV type 2a by mini-sequencing even using the faint PCR amplicons. To confirm the mini-sequencing results, these three samples were passaged in A72 cells for 5 times to amplify the virus. PCR product from the 5th passage samples were then confirmed as CPV type 2a by cycle sequencing also. Therefore, mini-sequencing is much more sensitive technique than cycle sequencing in typing the CPV from direct fecal samples.

Cycle sequencing was performed using purified PCR products whereas the mini-sequencing was performed directly from the PCR amplicons. In some cases, cycle sequencing required pooling of multiple PCR reactions of a sample, whereas mini-sequencing was always performed using a single reaction of PCR product. Therefore, every sample can be subjected to mini sequencing whereas the

tedious cycle sequencing is generally performed for representative samples only. Since only specific SNPs useful in differentiating the types were involved in the mini-sequencing reaction, the analysis of result is simpler and rapid compared to the tedious sequence analysis to determine the types by cycle sequencing. Therefore, the mini-sequencing method is a sensitive, rapid and high throughput method to type CPV samples directly from suspected animal samples in a single tube.

In conclusion, the mini-sequencing technique can be used as a routine CPV typing method. The tedious virus isolation steps can be avoided as the typing is done from the clinical samples directly and several samples can be typed simultaneously. The method can also be used for the genetic characterisation of other viruses which vary by SNPs.

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