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Short Communication

SEQUENCE ANALYSIS OF VP2 GENE SEGMENT OF CANINE PARVOVIRUS ISOLATE FROM ANAND

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ABSTRACT
Canine parvovirus is the major cause of severe gastroenteritis in dogs characterized by depression, loss of appetite, vomiting, diarrhoea, myocarditis and is responsible for significant morbidity and mortality. Since the emergence of CPV-2 in 1978, a number of outbreaks have been reported due to the development of new variants, namely: CPV-2a, CPV-2b and CPV-2c. Increasing incidences of CPV in vaccinated dog population is also a matter of concern with regards to the prevailing CPV strains showing possible mutational escape from the vaccine strain. In the present paper, we have examined isolates of CPV from clinical samples of suspected dogs collected and fragment of the VP2 gene of the virus was analyzed using polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and DNA sequence analysis. We found that, eleven out of fourteen samples from dogs with diarrhoea were characterized as CPV-2c, out of which 5 samples were from vaccinated dogs, indicating that this virus is already circulating in the Indian canine population.

KEYWORDS: *CPV-2c; restriction fragment length polymorphism, DNA sequence.*

INTRODUCTION

Canine parvovirus was emerged in 1978, with the original isolate being termed as CPV type 2 (CPV-2). In 1979, a variant CPV strain designated CPV type 2a (CPV-2a) started to become widespread. CPV-2a differs from the original type 2 in five amino acid (aa) changes in the VP2 coat protein. The present evidence indicates that those aa are responsible for the antigenic and host-range viral properties (Truyen *et al*., 1995). In 1984, another antigenic variant emerged as a new CPV type, designated CPV-2b, which is currently co-circulating with the CPV-2a within dog populations around the world. The antigenic differences observed in CPV-2b are consequence of only one aa substitution (Asn426Asp) placed in the major antigenic site of the capsid (epitope A). More recently in 2001, strains have emerged in Italy in which the amino acid at position 426 (Asn in 2a and Asp in 2b) has been replaced by glutamic acid (Glu) residue (Martella *et al*., 2004). Since then, Glu426 variants termed as 'CPV-2c viruses', also circulate and coexist in Spain (Decaro *et al*., 2006), Vietnam (Nakamura *et al*, 2004), Germany (Shackelton *et al*., 2005), United Kingdom and other European countries (Decaro *et al*., 2007), South America (Perez *et al*., 2007) and Argentina (Calderon *et al*., 2009). The Asp → Glu change at residue 426 of CPV-2c strain was due to a change (T → A) in the third codon position, at nucleotide 4064. Since the nucleotide variation at residue 4064 of CPV-2c strain created an *Mbo*II restriction site (GAAGA) unique to these strains, it is possible to distinguish these mutants from the other antigenic types *viz.*, from CPV type 2, 2a and 2b by simple digestion by *Mbo*II (Buonavoglia *et al*., 2001; Perez *et al*., 2007). Types 2a, 2b and 2c CPVs differ for the presence of one or two single nucleotide polymorphisms (SNPs) in the sequence of the capsid protein (VP2) gene. SNPs at positions 4062 and 4064 of the viral genome determine the presence of the capsid protein with amino acids Asn, Asp and Glu in types 2a, 2b and 2c, respectively, at residue 426 (Buonavoglia *et al*., 2001).The emergency and spread of CPV-2c is considered a sanitary threat worldwide. The monitoring of CPV field isolates has been fundamental to understand the virus epidemiology and to develop preventive measures (Perez *et al*., 2007).The present study describes the presence of type 2c in vaccinated dogs in Anand district of Gujarat. The findings support worldwide spreading of this new type and provide new information to understand the evolution of antigenic variants of CPV.

MATERIALS AND METHODS

Twenty rectal swabs, from vaccinated and non vaccinated dogs (one to six months of age) suffering from diarrhoea, were collected from November 2009 to March 2010. The samples were obtained from Zaveri clinic and veterinary dispensaries in Anand district.

DNA extraction and PCR amplification

DNA extraction was performed according to the method described by 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. Nobivac® puppy DP vaccine was resuspended in 1 ml of PBS and then processed using the same methodology.

PCR was carried out according to previous study (Pereira *et al*., 2000), using primer pairs *Pbs/Pbas* (CPV-2b specific), that amplifies a 427 base pairs (bp) fragment of

the VP2 gene (position 4043 - 4470). 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.

RFLP

All the samples, including CPV-2 type vaccine, were subjected to RFLP assay. The PCR products were digested with five units of the *Mbo*II restriction enzyme (Fermentas) and observed on 2% agarose gel to determine cleavage patterns.

Sequencing

The positive PCR products, which were from vaccinated dogs and showing digestion, as well as CPV-type 2 vaccine were purified and sequenced using an ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems, USA), after being cloned using the InsT/Aclone PCR product cloning kit (Fermentas). Sequencing was carried out on both strands using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Sequence analysis

In order to compare the field isolate with other CPV strain sequence, published sequences were retrieved from the GenBank. Nucleotide and aa sequence alignment was performed by ClustalW method with BioEdit Sequence Alignment Editor (http://www.mbio.ncsu. edu/BioEdit/ bioedit.html) and phylogenetic analysis was also carried out. The GenBank accession numbers of the nucleotide sequences of reference CPV strains used for comparison are as follows: CPV-b variant (reference CPV-2, M38245); CPV-15 variant (reference CPV-2a, M24003); CPV-39 variant (reference CPV-2b, M74849). The comparative sequence analysis was also performed with the reported sequences of Indian CPV isolates from Maharashtra (DQ182616), New Delhi (DQ182622), Hyderabad (DQ182627), Uttar Pradesh (AJ698134) and Manipur (EU118267), and foreign CPV -2c isolates from Italy (AY380577), Germany (AY742942), Vietnam (AB120727), Brazil (EU797728), Portugal (EU273771), USA (FJ005236) and Argentina (FJ349322).

RESULTS

A single band of expected size (427 bp) was observed by gel electrophoresis in fourteen of the twenty field samples suspected of having CPV. A band of same size was amplified from CPV-type 2 vaccine and used as positive control. The negative samples were assayed twice to check for negative results. In order to test for the presence of CPV-2c in the PCR positive samples, we performed a RFLP analysis using the *Mbo*II enzyme, as reported by Buonavoglia *et al.* (2001). Our analysis showed 11 out of 14 positive samples with type 2c RFLP pattern and three samples had a CPV-2b pattern, while vaccine remained undigested. Among the positive samples, PCR products of five out of 11 samples which were obtained from vaccinated dogs and showing digestion, were sequenced. All the sequences from the field samples were exactly (100%) similar and hence, a representative sequence was used for further analysis. The vaccine and field sequence from vaccinated dogs, were submitted to GenBank with accession numbers and compared with GenBank sequences of CPV-2, CPV-2a, CPV-2b and other CPV-2c using a ClustalW based online sequence alignment tool. Based on the occurrence of the codon GAA at position 4062 to 4064, which encodes for glutamine at residue 426, field isolate was confirmed as CPV-2c. The comparative sequence analysis was performed using a 427 amplicon that codes for 142 aa of the VP2 protein. The amino acid sequence of Anand isolate showed Glutamate at 426th residue which again suggests CPV- 2c strain.

DISCUSSION & CONCLUSIONS

In the present study, eleven out of fourteen samples were type 2c, out of which five samples were from vaccinated dogs. The presence of CPV gastroenteritis in vaccinated animals has been reported previously (Deepa & Saseendranath, 2000). With an increasing number of cases of sick dogs showing symptoms suggestive of CPV, including vaccinated animals, raises concerns among breeders, owners and veterinary practitioners about the ability of the current vaccines to protect the pups, as noted previously in other countries (Decaro *et al*., 2008; Lamm & Rezabek, 2008). In the previous years, scientists from many countries analyzed CPV samples to determine the circulating types and found high frequency of the type 2c (Martella *et al*., 2005; Vieira *et al*., 2008). Probably, this new viral type could have some adaptive advantage that leads it to replace the types 2a and 2b. It has been reported that, compared with other DNA viruses, CPV shows a higher rate of nucleotide substitution during replication, being similar to that reported for RNA viruses (Shackelton *et al*., 2005). This high mutation rate along with selective pressure, which might be generated by suboptimal levels of neutralizing antibodies due to incomplete or improper vaccination of dogs, might contribute to the emergence of new CPV variants.

Previously, the canine parvovirus types 2a and 2b had completely replaced the type 2 worldwide. Chinchkar *et al.* (2006) studied the epidemiology of CPV infections in dogs in India and found that CPV-2a isolates were predominant over the CPV-2b variant in central and southern India. Comparison of the VP2 gene sequences revealed that the Indian isolates formed separate lineages distinct from the South-East Asian isolates. The Indian CPV isolates appeared to evolve independently and distinct geographic pattern of evolution was not identified in the isolates. However, the type 2c samples analyzed in the present study were identical to Italy and Germany CPV strains showing 100 per cent sequence homology; hence it indicates that the Anand isolate does not have independent origin. However, more samples from other geographic regions of India as well as world are necessary to have better understanding of evolution of CPV genotypes.

Nandi *et al.* (2007) studied that CPV-2b is the predominant strain in dogs suffering from CPV in northern India. As we obtained 11 out of 14 samples to be CPV-2c (by PCR-RFLP), we can hypothesize that the CPV-2c is now replacing the previously circulating CPV strains, as occurred in Italy. After its first occurrence, the Italian CPV-2c increased its proportion in relation to other

circulating types from 17 per cent in the year 2000, to 60 per cent in 2004 (Martella *et al*., 2005). This result together with our findings indicates that the spreading ability of the type 2c is associated with an important replacement capacity that could eventually lead to the elimination of other CPV types. Our samples also had Valine instead of Isoleucine at position 555, as a consequence of single transition (A/G) at nucleotide 4449. This change is considered a reversion to the original CPV-2 and it has been reported in most recent 2a isolates. However, this reversion also occurred in prevalent isolates as observed in Indian samples by Chinchkar *et al*. (2006). The CPV-2a strain probably represents the relic of the original types that were possibly predominant in Anand area before the CPV-2c appearance. With this sense, the previous population would be typical CPV-2a as reported by Chinchkar *et al.* (2006).

The comparison of gene sequences among different variants revealed that major part of the gene is conserved. However, changes at specific nucleotide position affect the major antigenic site of the viral capsid that determines the unique variant of CPV. The efficiency of the current vaccines against this mutant is another question that must be addressed. There is only one report which shows complete protection against type 2c by an attenuated vaccine based on other CPV type (Spibey *et al*., 2008). On the other hand, there are several works reporting vaccine failures when the challenge virus is of the type 2c (Decaro *et al*., 2008; Perez *et al*., 2007). In order to obtain better protection against the field strains of CPV, the incorporation of a specific new variant of CPV-2 in the vaccine is recommended based on the prevalence in the country.

All the puppies sampled had clinical signs of gastroenteritis. Differences in clinical signs induced by distinct viral types have been reported (Perez *et al*., 2007; Carmichael, 2005). In India, since the first report of CPV-2 by Ramadass & Khader (1982), numerous CPV outbreaks in dogs have been reported which showed different clinical signs especially over the last few years from different parts of the country (Phukan *et al*., 2004; Biswas *et al*., 2006). It is important to have detailed information on the genetic makeup of the CPV strains used in the commercial vaccines.The present study has given an insight into the ever-changing genetic makeup of CPV, and probably addresses the CPV vaccine failures in field conditions. The finding of CPV type 2c in field samples demands a continuous scientific effort in the country to monitor and characterize the circulating CPV so as to get rid off this important disease menace faced by canine population.

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