

Phylogenetic analysis of the partial VP2 gene of canine parvovirus-2 from the northern region of India

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ABSTRACT

Canine parvovirus-2 (CPV-2) infection is a serious problem causing a high rate of mortality in puppies. Despite the widespread vaccination of domestic dogs, a major impediment in the control of this deadly disease is the presence of different antigenic variants in field. Regular surveillance and constant monitoring of these variants, which might evade the host immune pressure and laboratory detection, is critically essential. Thus, the present study was aimed at understanding the molecular epidemiology of CPV-2 strains circulating in northern region of India. Polymerase Chain Reaction (PCR) positive samples were subjected to oligonucleotide sequencing and these isolates were found to be identical to CPV-2a except at positions 264, 297 and 440 amino acid residue, and thus typed as an antigenic variant of CPV-2a. The mutation at position 264 has not been reported from India before. Furthermore, global phylogenetic analysis confirmed the molecular relationship of these new CPV-2a isolates with sequences from China.

Key words: CPV-2, polymerase chain reaction, antigenic variants

Introduction

Canine parvovirus type 2 (CPV-2) is a non-enveloped single stranded DNA virus (5.2 kb), causing high mortality in young non-immune dogs aged from 6 weeks to 6 months (BAGSHAW et al., 2014). The clinical signs include anorexia, fever, hemorrhagic diarrhea and fever. Canine parvovirus type 2 (CPV-2) is a type species of the genus *Parvovirus* that belongs to the family *Parvoviridae* under the sub-family *Parvovirinae*. CPV-2 was first identified in early 1978 as a pathogen causing acute hemorrhagic enteritis and leucopenia in young dogs (APPEL et al., 2011).

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Canine parvovirus-2 is believed to originate as a host range variant from the feline panleucopenia virus (FPLV), through a small number of mutations in the single VP2 gene (ELIA et al., 1979). Later, many other antigenic variants, such as CPV-2a (1980), CPV-2b (1984) and CPV- 2c (2000), began appearing in the canine population and replaced the original type 2 virus (TRUYEN, 2006; PRATELLI et al., 2000; BUONAVOGLIA et al., 2001). Amino acid substitutions at positions 87 (Met to Leu), 300 (Ala to Gly) and 305 (Asp to Tyr) led to the evolution of 2 to 2a and substitution at 426 (Asn to Asp) and 555 (Ile to Val), giving rise to the emergence of 2b from 2a (SPIBEY et al., 2000). CPV-2c has a different amino acid, glutamic acid at position 426 compared to CPV-2b (MARTELLA et al., 2001). Apart from these classical CPV-2 variants, many mutations, such as Ser297Ala, Thr440Ala and Tyr324Ile have been reported from India and all over the world (MITTAL et al., 2014; CHINCHKAR et al., 2006; DECARO et al., 2006). Many authors have reported that canine parvovirus type 2a/2b having a mutation at 297 residue (Ser 297 Ala) is designated as New CPV-2a/2b (DECARO et al., 2006; OHSHIMA et al., 2008; RAJ et al., 2010).

The present study was aimed at sequencing of CPV-2 positive samples collected from the polyclinic of the Indian Veterinary Research Institute, and identification of any changes in the genetic composition of local isolates from its original prototype, by phylogenetic analysis.

Materials and methods

Selection of samples and oligonucleotide sequencing. As mentioned in our previous report (THOMAS et al., 2014), 23 dog fecal samples were confirmed to be positive for CPV-2 out of total 44 samples collected from diarrheic dogs presented to the veterinary polyclinic of the Indian Veterinary Research Institute, Bareilly. Bulk amplification of the randomly selected 15 PCR positive samples, targeting the partial VP2 gene was done as described previously (THOMAS et al., 2014) and 564 base pair products were purified using an Invitrogen Purelink PCR purification kit (Thermoscientific, Carlsban, USA), and custom sequenced using the primer pair CPV-PF/CPV-PR.

Phylogenetic analysis. The specificity of the obtained sequences was determined using a Basic local Alignment Search Tool (BLAST) and the further sequence chromatogram was visualized by BioEdit v 7.0.5 analysis software (Isis Therapeutics, Carlsbad, CA, USA) for proper alignment. All the sequences were aligned with the corresponding sequences available in the Gene Bank by the Clustal W (Mega5 software) method (TAMURA et al., 2011), by considering all the critical amino acid residues on the VP2 capsid protein (297, 300, 305 and 426). The assembled sequences were also submitted to NCBI for allotment of accession numbers (<http://www.ncbi.pubmed>). The obtained sequence in this study and VP2 gene sequences of other reference CPV-2 variants from different geographical regions within India (eastern, western, southern and northern India) and the rest of the

world were retrieved from the NCBI nucleotide database (Total number of retrieved sequences-81). Multiple sequence alignment was performed using the in-built ClustalW algorithm in MEGA6 software. Selection of the best-fit nucleotide substitution model for the data set confined to the partial VP2 gene (564 bp) was conducted in MEGA6. The evolutionary history was inferred using the Neighbor joining method, based on the p-distance model. The confidence level of branching in the phylogenetic tree was evaluated with the bootstrap test based on 2,000 resamplings.

Results

A region over the VP2 gene, from nucleotide positions 772 to 1335 (amino acid positions from 258 to 445), was selected for bulk amplification using CPV-PF/CPV-PR primers, which yielded a specific 564 bp product, as described previously (THOMAS et al., 2014). Amplified products of positive samples were sequenced and aligned by the Clustal W (Mega5 software) method. The assembled sequences were assigned by Gene Bank accession numbers from KJ-364526 (BE-3) and KM-003870 to KM003883 (BE-4 to BE-17) and revealed that all the examined sequences significantly matched CPV-2a, with a maximum of 99% identity with the VP2 gene sequences of the reference strain (accession number = EU377537) available in the Gene Bank. Phylogenetic analyses of isolates with other Indian sequences and foreign sequences are depicted in Fig.1 and 2 respectively.

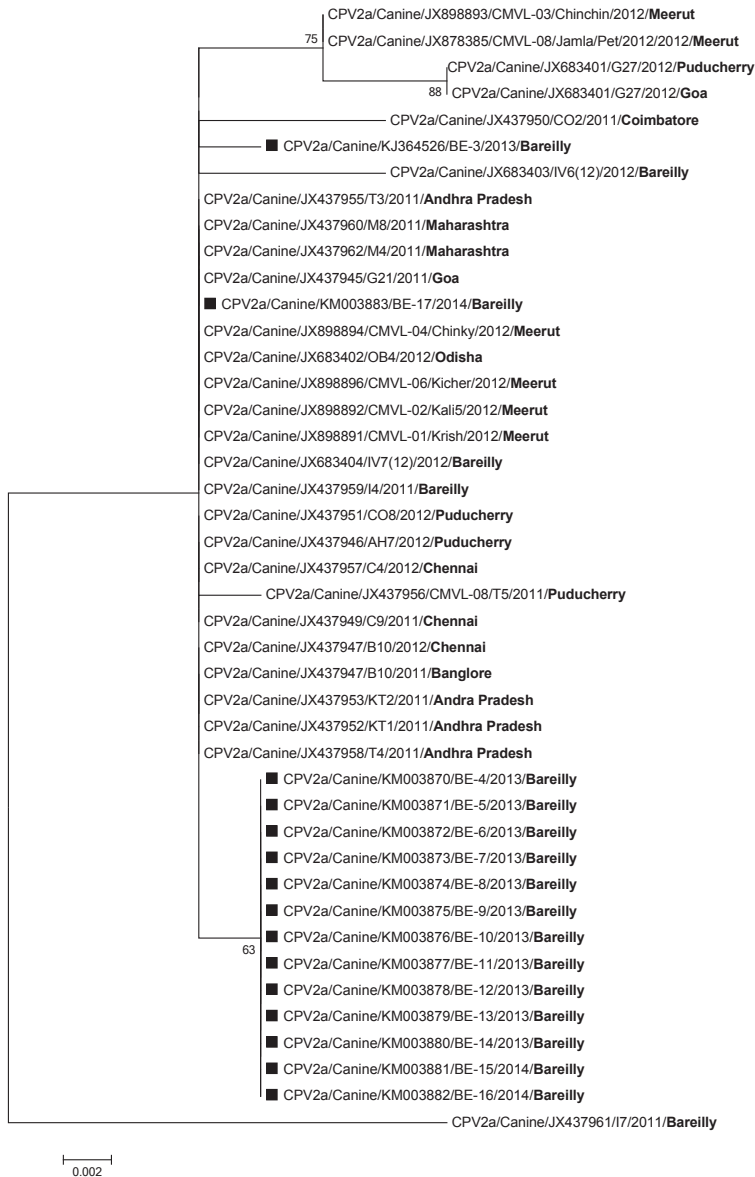


Fig. 1. Neighbour joining tree depicting phylogenetic relationship between isolates of the present study with various CPV-2a isolates from different parts of India

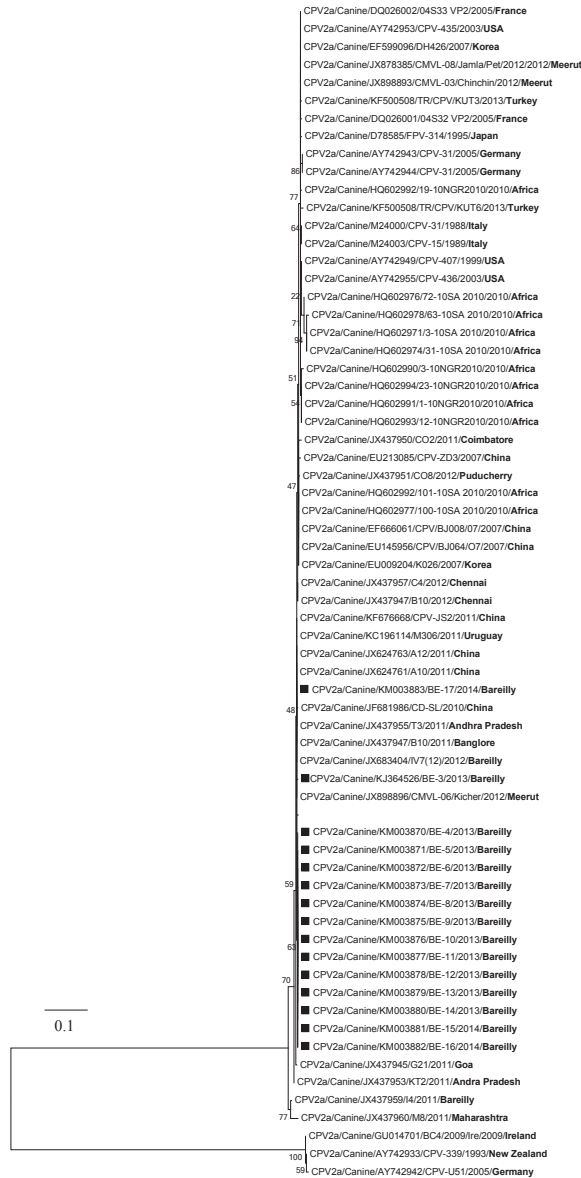


Fig. 2. Neighbour joining tree depicting phylogenetic relationship between isolates of the present study with various CPV-2a isolates from India and other countries. The sequences in the present study are marked with bullet.

Discussion

The region selected for amplification contains 188 amino acids (564 nucleotides), which includes almost all informative amino acids responsible for generation of different antigenic variants reported so far. Thus, the amplification of the partial VP2 gene covering the key amino acids, using primers CPV-PF/CPV-PR, and its oligonucleotide sequencing may be helpful in identification of CPV-2 variants. There are several methods available for the molecular characterization of CPV variants, however, PCR amplification of the VP2 gene and subsequently oligonucleotide sequencing of the amplified product is considered to be a gold standard for identification of any antigenic or genetic differences between the original CPV-2 and its variants (NANDI and KUMAR, 2010). The finding of maximum prevalence of CPV-2a in India is contrary to another report (NANDI et al., 2010) where it was reported that CPV-2b is the major antigenic variant in North India, especially in the Bareilly region. Although some more samples from diverse sources should be examined before concluding that CPV-2a is now outpacing CPV-2b in this region, this seems to be the normal trend of distribution, as it has been demonstrated in some previous reports that CPV-2a is more common than CPV-2b in the Indian subcontinent (MUKHOPADHYAY et al., 2014; CHINCHKAR et al., 2006).

CPV-2 regularly changes its genetic and antigenic character through frequent mutations in the VP2 gene, and the resulting variants (CPV 2a/b/c) are spreading throughout the world (NANDI et al., 2009). In India, it has been reported that CPV-2a is the major antigenic variant of CPV-2, followed by CPV-2b (MUKHOPADHYAY et al., 2014). Recently MITTAL et al. (2014) reported some CPV-2a variants with amino acid mutations at positions 297 (Ser297Ala), 324 (Ile 324 Tyr) and 440 (Thr 440 Ala). In the present work, we examined patterns of nucleotide sequences of 15 randomly selected samples out of 23 PCR positive samples available in the laboratory, and the sequencing data revealed Glycine at position 300, Tyrosine at 305 and Asparagine at 426. This suggests that all the sequences are of the CPV-2a type. The amino acid residues at positions 87, 101 and 555 are unknown because these regions are not covered by the partial VP2 gene selected for PCR amplification. However, the deduced sequences of CPV-2 isolates in the present study showed some additional nucleotide substitution that resulted in the amino acid variations at positions 264 (Gly264Val), 297 (Ser297Ala) and 440 (Thr440Ala). The amino acid substitution at position 264 was observed only in 10 sequences because 20 to 30 nucleotides were missing from the 5' end of the remaining 4 sequences due to a sequencing artifact, and there was no nucleotide substitution recorded in BE-3 isolates at this position. Both these mutations were also reported from China in 2011 (Gene accession number- JF681986.1). Mutation at 297 and 440 amino acid residues has already been reported in CPV-2a strains from India by MITTAL et al. (2014) and CHINCHKAR et al. (2006). However, the mutation at the 264 amino acid residue has not yet been reported in India. The greatest variability

among CPV isolates were detected in the GH loop of the VP2 protein, particularly at 297, 426, and 440 amino acid residue, and mutations at these regions are indicative of some new antigenic variants evolving in the circulating CPV types (PARRISH et al., 1991). The sequencing results in the present study reveal change at the 297 position of the VP2 capsid protein, which is essentially required for considering any isolates as a new CPV-2a/2b. Moreover the mutation at position 440 also has antigenic importance, as it is located in the GH loop of the VP2 protein on the surface of the capsid (BATTILANI et al., 2002). The mutation at position 264 is very rare and has not been reported previously from India. It indicates that a new CPV-2a variant is spreading in this region with additional mutation at position 264 which has never been reported before.

Sequence analysis of three vaccinated but PCR positive dog fecal samples (BE-11, BE-12, BE-14) shows that all are of the CPV-2a type, with a few additional synonymous mutations which exclude the possibility of the shedding of vaccine strains through feces in the current study, because none of the commercially available vaccines contains the CPV-2a strain in its formulation. Hence, the reason for this vaccination failure may be either lack of a proper vaccine strain, improper dosing, or faulty administration.

Constructing the phylogenetic tree on the basis of deduced nucleotide sequences is an ideal way to find the origin and source of CPV-2. The amount of genetic change that occurred in the phylogenetic analysis of the isolates compared with other Indian sequences is 0.002, and with foreign sequences 0.05. This indicates that the genetic change between the Indian sequences is much less than when compared with foreign sequences. The VP2 gene is relatively constant and thus conclusions about separate lineages should be moderated. However, most of the new CPV-2a isolates, except BE-3 and BE-17, clustered in the same monophyletic clade in the Neighbor joining analysis at the region of the VP2 gene. BE-17 forms a separate lineage with other new CPV-2a isolates in the present study, while BE-3 forms a distinct lineage. This may be due to the presence of thymine at nucleotide position 3978 in both BE-3 and BE-17, instead of cytosine in other isolates, and there was no mutation recorded at position 264 in BE-3 when comparing with other isolates in the study. BE-3 and BE-17 show more similarity with sequences from north (Bareilly, Meerut), east (Odisha) and southern regions (Andhra Pradesh, Chennai and Puducherry) of India than other isolates. All the isolates in the present study, especially BE-3 and BE-17, share common ancestral origin with sequences from China. Hence, the isolates from the northern region of India used in the present study might have their origin in China, and this is similar to the findings observed by MUKHOPADHYAY et al., (2014).

Conclusion

Despite regular vaccinations, CPV-2 infection is still a great threat to the canine population in India and across the world. The only way forward is to continuously

monitor the CPV-2 variants circulating in this region, which would be helpful in the identification of any major or minor changes occurring in the CPV-2 genome. Further work in this direction, involving samples from a large geographical area, may explore the present finding of CPV-2 variants which would be extremely useful in understanding the role of these variants in the pathogenesis of parvoviral disease in dogs, and formulating a suitable control strategy.

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SAŽETAK

Infekcija pasjim parvovirusom 2 predstavlja ozbiljan problem uzrokujući veliki mortalitet u štenadi. Usprkos proširenom cijepljenju pasa, veliku poteškoću u kontroli ove smrtonosne bolesti predstavljaju različite antigenske varijante terenskih izolata virusa. Od bitnog je značenja redoviti nadzor i trajna kontrola tih varijanti koje mogu izbjeći imunski pritisak domaćina i otežati laboratorijsku dijagnostiku. Stoga ovo istraživanje ima za cilj rasvijetliti molekularnu epizootologiju sojeva pasjeg parvovirusa 2 koji kolaju na sjevernom području

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Indije. Uzorci pozitivni lančanom reakcijom polimerazom podvrgnuti su oligonukleotidnom sekvencioniranju. Ustanovljeno je da su ti izolati bili identični pasjem parvovirusu 2a, osim na mjestima aminokiselinskog ostatka 264, 297 i 440 što im upravo određuje pripadnost serotipu 2a. Mutacije na mjestu 264 dosada nisu bile opisane u Indiji. Globalnim filogenetskim analizama utvrđena je molekularna srodnost tih novih izolata pasjeg parvovirusa 2a s izolatima iz Kine.

Ključne riječi: pasji parvovirus 2, lančana reakcija polimerazom, antigenske varijante
