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Genomic typing and phylogenetic analysis of canine parvovirus detected in the state of Odisha, India

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ABSTRACT

Canine parvovirus type 2 (CPV-2) comprises three major antigenic variants CPV-2a, CPV-2b and CPV-2c. Their mutated variants in geographically distinct locations need to be investigated to understand viral evolution and for development of effective management measures. In the present study, 71 faecal and 12 blood samples from suspected dogs in the state of Odisha, India were analyzed by PCR. Faecal lysate, extracted by the fast boiling method was found to be more sensitive as a template for PCR compared to DNA extracted from faecal samples by the phenol-chloroform method. The results revealed 29 positive cases (583 bp amplicon) out of 71 faecal samples, and 5 positive cases out of 12 blood samples examined, with a few variations in the results from blood and faecal samples in the same cases, thus suggesting the necessity of screening both blood and faecal samples for diagnosis. Restriction digestion of the 583 bp PCR amplicon with *Mbo*II (PCR-RFLP) confirmed the strain not to be CPV-2c. Further sequencing of the 583 bp fragments recognized the variant as one of the mutated CPV-2a strain. Interestingly, an additional presence of CPV-2a mutant of 525 bp was observed in eleven

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of the positive faecal samples, along with the 583 bp fragment in PCR that needs further characterization. These two CPV-2a variants shared a common clade with other CPV-2a variants in the phylogenetic tree separating CPV-2b and CPV-2c. Our results confirm the dynamic changes in CPV variants and emphasize the importance of CPV surveillance for understanding of viral epidemiology.

Key words: canine parvovirus, diagnosis, PCR, new variant

Introduction

Canine parvovirus (CPV) is a nonenveloped, icosahedral, single stranded DNA virus of 5. 2 kb which encodes two nonstructural proteins (NS1 and NS2) and two structural proteins (VP1 and VP2) (REED et al., 1988). VP2 is the major capsid protein which is highly antigenic. There are two antigenic variants of CPV: CPV-1 and CPV-2. Emergence of newer variants of CPV-2, such as CPV-2a, CPV-2b and CPV-2c are prevalent with a mutation rate $1x10^{-4}$ to $4x10^{-4}$ changes/nt/year (BUONAVOGLIA et al., 2001). Although all the variants of CPV-2 are endemic in most populations of domestic and wild canids, CPV-2a has now become widespread world-wide (PARRISH et al., 1985; PARRISH et al., 1991; MITTAL et al., 2014; MUKHOPADHYAY et al., 2014; CALDERON et al., 2015; WU et al., 2015; MIRANDA et al., 2016; WANG et al., 2016). CPV-2a differs from CPV-2 in five amino acids (aa) in VP2 resulting in a change in one epitope, which is more specific, as recognized by monoclonal antibodies (MAbs). The incidence of CPV-2 variants has been reported from various states, such as: Kerala, Odisha, Assam, West Bengal, Tamil Nadu, Pondicherry, Haryana, Andhra Pradesh, Karnataka, Maharashtra, Goa and Uttar Pradesh (MUKHOPADHYAY et al., 2014; NANDI and KUMAR, 2014). Studies of infected dogs from different parts of India, such as the states of Maharashtra, Gujarat, Uttar Pradesh, New Delhi and Andhra Pradesh, have revealed the CPV-2a variant as the most common circulating strain in India, apart from a few cases of CPV-2b (CHINCHKAR et al., 2006; MUKHOPADHYAY et al., 2014). However, scarce literature is available on the circulating strains of CPV in the eastern part of India, particularly for the state of Odisha.

The presumptive diagnosis of this infection based on clinical signs is not definitive, as several other pathogenic organisms also mimic the same pattern of clinical signs, such as fever, vomiting, bloody diarrhoea, dehydration and significant leucopenia. Therefore, diagnosis must be based on confirmation by use of various laboratory methods. The different methods used for detection of the virus include electron microscopy (JOSHI et al., 1997), ELISA (PHUKAN et al., 2005), immunochromatographic test, a haemagglutination test, a haemagglutination inhibition test, polymerase chain reaction (PCR), loop-mediated isothermal amplification, insulated isothermal PCR, recombinase polymerase amplification (WANG et al., 2016a) and quantitative PCR (qPCR) based detection. The qPCR assay is more preferable in terms of quantitative detection of the virus and nowadays genotyping of variants is even easier, with the specific primers containing the mutants that identify

the variants (DECARO and BUONAVOGLIA, 2012). However, taking the mutation rate of this virus into consideration, PCR amplification and subsequent sequencing seem to be more preferable, as they are able to identify the additional mutations or variants emerging in the environment. Besides, PCR has already been successfully applied to detect human parvovirus (KOCH and ADLER, 1990), porcine parvovirus (PPV) (MOLITOR et al., 1991) and many important animal pathogens (BELAK and BALLAGI-PORDANY, 1993).

Most of the diagnostics were conducted using faecal samples as a rich source of viral particles (MUKHOPADHYAY et al., 2014; CALDERON et al., 2015). According to the pathogenesis of the virus post-entry through the oropharynx, initial viral replication occurs in the pharyngeal lymphoid tissue. Then the virus is distributed to other organs through the blood stream (MURPHY et al., 1999; MacLACHLAN and DUBOVI, 2011). Clinical cases mostly arrive at the clinics when the dogs are suffering from acute infections showing bloody diarrhea or towards the later part of the viraemia. Hence, testing of blood samples is also necessary to reveal the duration and severity of the infection. Again the type of sample preparation methods can also influence the sensitivity of viral particle detection based on the nature of the sample. Most researchers use either fast boiling or phenol-chloroform based methods for fecal sample preparation. Here an attempt was made in the study to look into whether either of these two methods affect the final diagnosis, by analyzing faecal samples using both methods.

Taking all these above into consideration, attempts were made in the present study to diagnose CPV by identification of the viral strain through PCR-based screening using both faecal and blood samples from infected dogs found in the eastern part of India, in the state of Odisha, along with its strain characterization.

Materials and methods

Collection of samples. Dogs from various parts of Odisha, from places such as Berhampur, Sambalpur, Jajpur, Khurdha and in and around Bhubaneswar, mostly suffering from diarrhoea and vomiting, were selected for the study as suspected cases of parvovirus infection from the Teaching Veterinary Clinical Complex (TVCC, a centralized state hospital), the College of Veterinary Science and Animal Husbandry, Odisha University of Agriculture and Technology, Odisha. Faecal samples were collected from 71 dogs in 10% w/v PBS and 12 blood samples in EDTA-anticoagulant during the period from December 2012 to March 2013. The samples were either immediately processed or preserved at -20 °C until further use.

Efficiency of sample preparation for PCR-based detection. The faecal suspensions in PBS were processed by centrifuging at 1500×g for 10 min at 4 °C. The supernatant was collected in 2 mL collection tubes and further processed by two different methods: the fast boiling method and the phenol-chloroform extraction method. Initially, eight faecal

samples were processed by both methods for evaluation of the efficacy of each method before processing further samples. The commercial vaccine against CPV, Canigen DHPPi (Virbac) was used as the positive control after pre-treatment by the fast boiling method. The blood samples were processed according to the phenol-chloroform extraction method only.

Fast boiling method of lysate preparation. The supernatant (500 µL) was boiled for 10 min at 100 °C to inactivate the PCR inhibitors and kept on ice (SCHUNCK et al., 1995; UWATOKO et al., 1995) until further centrifugation at 1500×g for 5 min. The supernatant (lysate) was stored at -20 °C until further use as a source of the DNA template for PCR.

Phenol-chloroform extraction. DNA extraction from eight faecal as well as all blood samples (500 µL each) was carried out by the phenol-chloroform method (SAMBROOK et al., 1989). The quality and quantity of the extracted DNA was checked using NanoDrop ND 1000 (Thermo Scientific, USA) by checking the optical density at 260 and 280 nm.

PCR-based detection. PCR was set using CPV-2 specific primers (BUONAVOGLIA et al., 2001) with 5 µL of lysate or 10 µL of DNA as the template. Briefly, 0.5 µL of each 555 primer (forward - 5'CAGGAAGATATCCAGAAGGA3' and reverse -5'GGTGCTAGTTGATATGTAATAAACA-3'), 0. 5 µL of 2 mM dNTPs (Fermentas), 2. 5 µL of *Taq* DNA buffer, 0. 25 µL of *Taq* DNA polymerase (5 U/µL, Genei, Bangalore) were prepared in a total reaction volume of 25 µL. The reaction condition comprised initial denaturation at 94 °C for 2 min, then 40 cycles of denaturation at 94 °C for 45 seconds, annealing at 61 °C for 45 seconds, extension at 72 °C for 2 min and a final extension at 72 °C for 10 min. The amplified PCR products were analysed on 1% agarose gel (Lonza, USA) with the positive control, and visualized under a UV transilluminator (Alpha Innotech, USA).

Purification of amplified PCR products. The PCR amplicons showing more than one band were gel purified from 1. 5% low melting agarose (Lonza, USA) using a Genei pure gel extraction kit (Merck, Mumbai) in accordance with the manufacture's protocol. The PCR reaction which amplified the single band was directly purified using a Genei pure quick PCR purification kit, according to the manufacturer's instructions. The purified PCR products were quantified by running in agarose gel with a λ3 marker (Fermentas) and a 100 bp ladder.

Restriction enzyme mapping. Ten numbers of purified PCR products (desired fragment) were subjected to restriction digestion. The purified samples were digested with the enzyme *Mbo*II (Fermentas), whose restriction site was unique to the Glutamate 426 mutants, i.e. CPV type-2C (BUONAVOGLIA et al., 2001). To about 5 µL of the purified DNA sample, 1 µL of the restriction enzyme (*Mbo*II) was added with 1 µL of 10 × RE buffer B in a total reaction mixture of 10 µL, and incubated overnight at 37 °C. Then, the

obtained RE digested products were analysed in 1% agarose gel, along with the uncut PCR purified samples. The gel was visualized under the UV-transilluminator.

Sequence analysis. The fragments obtained in the PCR amplification were cloned in a pTZ57R/T-vector into DH5α *Escherichia coli* bacterial host using an InsTAclone PCR Cloning Kit (Thermo Scientific) according to the manufacturer's instructions. The positive clones were screened by restriction digestion with *Eco*RI and *Hind*III (Genei). Three of the positive clones were sequenced from three numbers of cases showing a single band and three numbers of cases showing double bands of three distant localities, and consensus sequences were derived. Consensus sequences were derived using the BioEdit 7. 0. 0 (PINTO et al., 2012) sequence alignment tool and the final sequences were submitted to GenBank. Along with the sequencing of amplicons obtained from faecal samples, the amplicons from the blood as well as the vaccine were also sequenced in order to rule out any false positive cases.

The two sequences (GenBank ID KF539788, KF539787) obtained were translated into amino acid sequences and aligned with the few available sequences of CPV-2 (M38245. 1), CPV-2a (M24000. 1, M24003. 1, FJ005256, GQ379048. 1, GQ169539. 1, GU380298. 1), CPV-2b (EU483517. 1, DQ182623. 1) and CPV-2c (GQ865519. 1, FJ222821. 1) using Clustal W multiple alignment, implemented in BioEdit version 7. 0. 0, to confirm the strain. A phylogenetic tree was constructed using 52 CPV2 sequences from eight countries (Taiwan, Italy, China, USA, South Korea, Greece, Thailand and Uruguay) and six regions of India (Uttar Pradesh, Tamil Nadu, Andhra Pradesh, Maharastra, New Delhi and Gujarat) along with the sequences of mink enteritis virus (M24001. 1) and feline panleukopenia virus (M38246. 1) using the neighbour-joining method in the bootstrap test of Molecular Evolutionary Genetics Analysis 4 (MEGA 4) software (KUMAR et al., 2008).

Results

Efficiency of sample preparation for the PCR based detection method. The eight samples processed initially by the two extraction methods showed that the fast boiling method was more suitable than phenol-chloroform extraction method. Although 5 µL of lysate was used in the PCR reaction as compared to 10 µL of phenol-chloroform extracted DNA (which is roughly estimated to contain 30 times more DNA) for diagnosis of the same samples, seven samples were positive in the lysate method as compared to six positive samples in the phenol-chloroform method out of the total eight samples screened. On the basis of this result, all the other 63 faecal samples were processed by the lysate method only.

Type of sample for virus detection. Out of 71 tested faecal samples, an amplicon of 583 bp was obtained in 29 (40.85%) samples, along with the CPV-2 type vaccine strain

which was used as the positive control, and 42 samples (59.15%) screened negative. Interestingly, another amplicon of 525 bp was observed in eleven of the positive faecal samples and others revealed a single band (i.e., 583 bp) (Fig. 1). Similarly, 583 bp products were also detected in 5 blood samples (41.66%) out of 12 samples. Due to the simultaneous examination of both blood and faecal samples from the same animal in 12 cases, that were collected at the same time from each animal, four different conditions were interpreted, that is: (1) both faecal and blood PCR positive in three cases, (2) faecal positive, blood negative in 3 cases (3) both faecal and blood negative in 4 cases, and (4) faecal negative, blood positive in 2 cases.

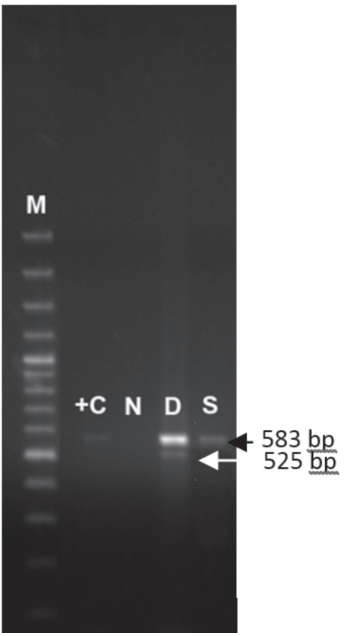

Fig. 1. PCR amplicons of faecal samples along with positive control [M: Molecular weight marker (100 bp), +C: Positive control, N: Negative sample, D: Positive sample with double band, S: Positive sample with single band].

Identification of the viral strain. The purified 583 bp fragments subjected to restriction digestion with *Mbo*II revealed a digested product of 566 bp in the gel (Fig. 2) (the smaller fragment of 17 bp was not visible). Since CPV-2c would have given fragments of 510 bp, 56 bp and 17 bp, the present strain was interpreted as either CPV-2a or CPV-2b. Further sequence analysis of the larger fragment of 583 bp confirmed the strain as CPV-2a (GenBank ID KF539788). The smaller fragment of 525 bp present in 11 samples was confirmed by sequencing as another variant of CPV-2a (GenBank ID KF539787).

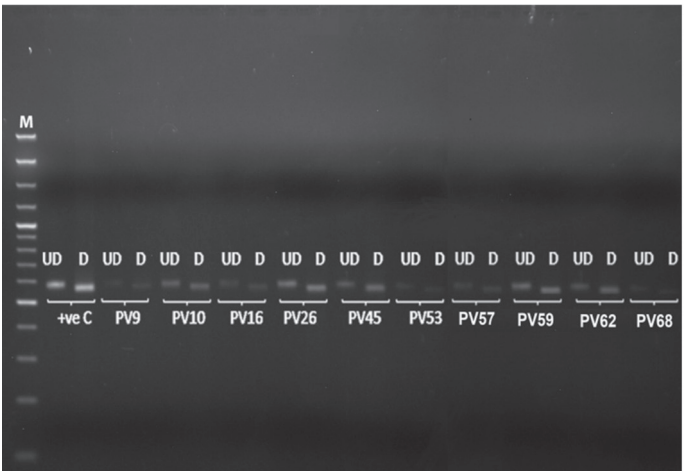

Fig. 2. Restriction digested (D) products along with its undigested PCR product (UD) of ten positive samples (larger fragment)

Table 1. Amino acid residues and substitutions in the VP2 gene of the CPV reference strain and isolates sequenced in the present study

Virus strain	Origin	Amino acid residues								
		426	440	541	555	574	575	577	578	579
CPV2_(M38245. 1)	USA	Asn	Thr	Ala	Val	Glu	Lys	Gln	Leu	Ala
CPV_2a_(M24000. 1)	USA	Asn	Thr	Ala	Ile	Glu	Lys	Gln	Leu	Ala
CPV_2a_(M24003. 1)	USA	Asn	Thr	Ala	Ile	Glu	Lys	Gln	Leu	Ala
CPV_2a_(FJ005256. 1)	Italy	Asn	Thr	Ala	Val	Glu	Lys	Gln	Leu	Ala
CPV_2a_(GQ379048. 1)	Thailand	Asn	Ala	Ala	Val	Glu	Lys	Gln	Leu	Ala
CPV_2a_(GQ169539. 1)	China	Asn	Ala	Ala	Val	Glu	Lys	Gln	Leu	Ala
CPV_2a_(GU380298. 1)	China	Asn	Ala	Ala	Val	Glu	Lys	Gln	Leu	Ala
CPV_2b_(EU483517. 1)	China	Asp	Thr	Ala	Val	Glu	Lys	Gln	Leu	Ala
CPV_2b_(DQ182623. 1)	India (New Delhi)	Asp	Ala	Ala	Val	Glu	Lys	Gln	Leu	Ala
CPV_2c_(GQ865519. 1)	Greece	Glu	Thr	Ala	Val	Glu	Lys	Gln	Leu	Ala
CPV_2c_(FJ222821. 1)	Italy	Glu	Thr	Ala	Val	Glu	Lys	Gln	Leu	Ala
CPV_2a_(KF539788)*	India (Odisha)	Asn	Ala	Ala	Val	Glu	Lys	Gln	Leu	Ala
CPV_2a_(KF539787)**	India (Odisha)	Asn	Ala	Gly	Val	Tyr	Ile	Thr	Ser	Thr

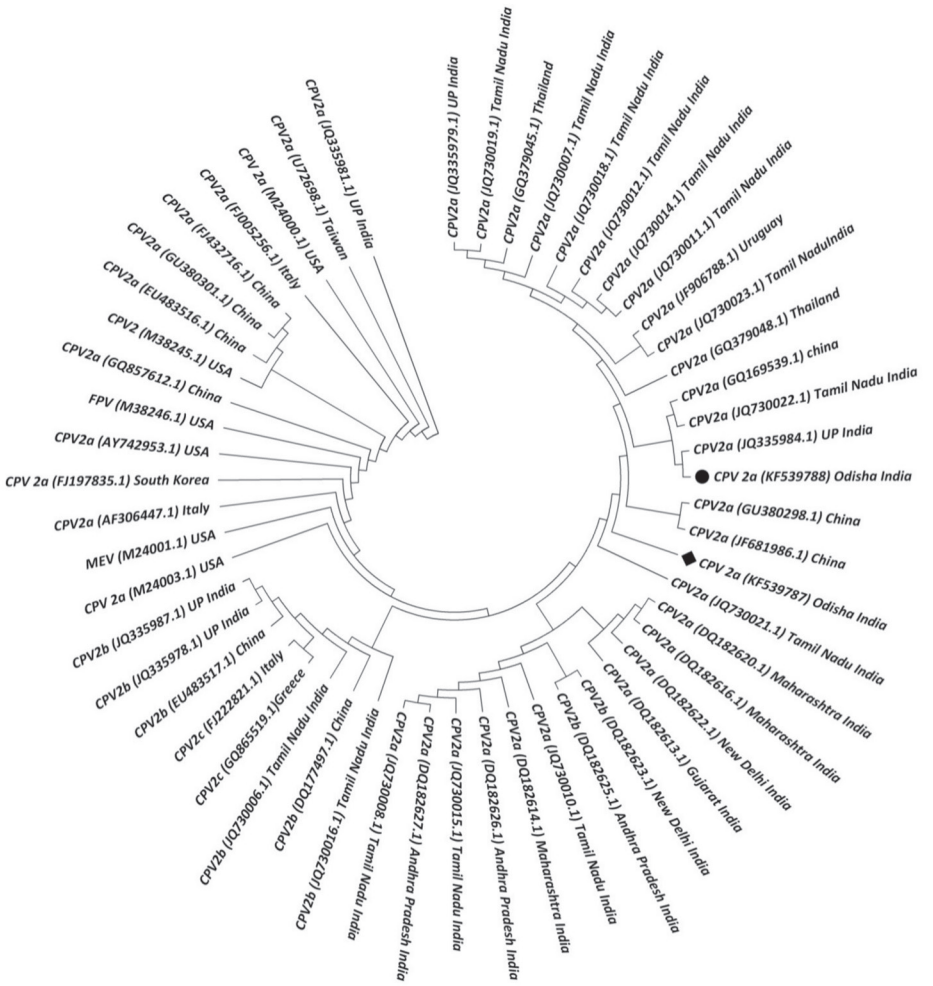

Fig. 3. Phylogenetic tree of CPV using VP2 gene of the obtained partial sequence information of Odisha strains (●,♦) along with other retrieved CPV sequences from GenBank. The tree was constructed using neighbor-joining method of MEGA 4. 0 software.

Sequence analysis. The translated amino acid sequences consisted of 178 amino acids for the larger fragment and 173 amino acids for the lower band of the CPV-2a variants. The alignment of these two fragments with other CPV-2 variants revealed the

presence of the amino acids asparagine (AAT), alanine (GCA) and valine (GTA) at 426, 440 and 555 positions, respectively, in both sequences. The codon AAT that codes for asparagine is present in CPV2/CPV2a strains. However, the presence of the remaining two characteristic residues at 440 and 555 permitted the accurate classification of the strain as CPV2a (DECARO and BUONAVOGLIA, 2012; PEREZ et al., 2012). The larger fragment had maximum similarity with two Chinese strains (GQ169539 and GU380298) and one Thailand strain (GQ379048) for 178 amino acids (Table. 1). The smaller fragment had a few additional mutations that have not been detected elsewhere. Sequencing of the smaller fragment showed similarity with CPV2a with an extra six transitions at 541 (Alanine to Glycine), 574 (Glutamic acid to Tyrosine), 575 (Lysine to Isolysine), 577 (Glutamine to Threonine), 578 (Leucine to Serine) and 579 (Alanine to Threonine) positions. Again it ended at the 579 position, unlike the upper band which ended at the 584 position. The sequence obtained from blood also matched the faecal derived larger fragment that was further different from the vaccine sequence (alanine at 440 position instead of threonine).

The phylogenetic analysis clustered all CPV-2a variants in one clade, and CPV-2b and 2c in another (Fig. 3). The Odisha strain, which was obtained from the larger fragment, clustered in the new CPV-2a clade and had maximum similarity to one variant from UP (India, JQ335984. 1) followed by Tamil Nadu (India, JQ730022. 1) and China (GQ169539. 1). However, the new strain of Odisha obtained from the smaller amplicon shared a separate branch in the same clade.

Discussion

PCR screening of 71 suspected faecal samples revealed 29 (40.85%) samples as positive for canine parvovirus. Similar incidences (42.29%) of parvovirus infection were reported earlier by PHUKAN et al. (2010) in Assam, India, by the sandwich ELISA method. In the present study, the fast boiling method of sample preparation was found to be more simple and advantageous than the phenol-chloroform extraction method. Similar results were obtained earlier (SCHUNCK et al., 1995; UWATOKO et al., 1995). The accuracy of the fast boiling method might be due to the fact that boiling eliminates or inactivates the inhibiting substances of *Taq* polymerase (SCHUNCK et al., 1995). However, the possibility of screening the blood samples with fast boiling method need further investigation. Three of the faecal and blood samples of the same animals, when screened by PCR, revealed positive result whereas four were negative in both samples by PCR. However, blood samples from vaccinated dog may lead to a false positive result. Therefore, it is necessary to take the vaccination history before taking blood sample into examination. In the present study, the sequences obtained from the blood positive samples matched the faecal positive sequences but not the vaccine sequence, which confirmed the detection of viral particles in the blood samples. Two the the blood samples were found to be positive where the

faecal samples were negative. The difference in the detection of the virus in faecal and blood samples may be due to the initiation of viraemia in the body which was detected in the blood before its appearance in the faeces. In contrast, three samples were positive in faecal materials which were not detected in the blood samples from the same animals. This may indicate a later stage of viraemia where the virus had been mostly/completely removed from the blood and was only present in the faeces. Therefore, blood samples can be used in diagnosis during the initial period of viraemia when faecal samples may show negative results. Hence, it is essential to screen both blood and faecal materials by PCR to diagnose this disease, since the presence and load of the virus in blood and faecal samples can provide confirmation of the presence, severity and duration of the infection.

Since its emergence, canine parvovirus 2 has undergone several mutations to develop many antigenic variants. CPV-2a and CPV-2b are more potent in developing variants which have fast spreading and replacement capacity (PARRISH et al., 1985; PARRISH et al., 1991) than CPV-2c, which showed a low level of genetic variability (DECARO et al., 2009). Further, no study has been undertaken in this part of the Indian state with proper characterization of the virus at a molecular level to determine the prevalent strain and the reason for vaccine failure. An earlier study carried out by BANJA et al. (2002) used only ELISA to confirm the parvo virus, but not at the strain level. Again the available vaccines were mainly of CPV-2 and CPV-2b. Therefore, the antigenicity of these variants needs to be studied to evaluate the usefulness of the available vaccines. Since the sequences of all three types of CPV-2 strains share a high level of homology, it is important to obtain the sequence information to identify the variants and their antigenic properties. Other diagnostic methods include *Mbo*II digestion of an amplified fragment using a common primer pair (555), which can amplify all three strains, but the digestion will affect only the CPV-2c strain (BUONAVOGLIA et al., 2001). However, the restriction map analysis of 583 bp fragments of three strains revealed a common *Mbo*II site in the 17th position, giving rise to two digested fragments of 17 bp and 566 bp in CPV-2a and 2b strains. CPV-2c contains an additional site at the 73rd position which gave digested products of 17 bp, 56 bp and 510 bp. In the present study, the digested product of 583 bp fragment showed a band of 566 bp (Fig. 2). The digested 17 bp fragment was not visible on the gel. This suggests that the present strain is CPV-2a or 2b, but not CPV-2c. Further sequencing of purified PCR products and alignment of the translated amino acid sequence with other CPV-2 variants revealed the studied strain to be CPV-2a. At position 426, asparagine was present which is a feature of both classical CPV-2 and CPV-2a. In the case of CPV-2b and CPV-2c this position is replaced with aspartic acid and glutamate, respectively (PARRISH et al., 1985). The presence of alanine at amino acid residue 440 is also a feature of the new CPV-2a variant (BATTILANI et al., 2002; CHINCHKAR et al., 2006; PEREIRA et al., 2007; PEREZ et al., 2012; MITTAL et al., 2014; WU et al., 2015) which is replaced with threonine in CPV-2. Transition at the 440th position may influence the antigenic properties since it

is located at the main antigenic site of the virus (TSAO et al., 2008) i. e. in the GH loop of the VP2 capsid protein. This residue is undergoing positive selection and has evolved into different populations independently, which explains its world-wide presence in unrelated CPV-2 populations (DECARO and BUONAVOGLIA, 2012; WU et al., 2015). However, it has not been detected by other researchers from India (MOHAN RAJ et al., 2010; KUMAR and NANDI, 2010). Nevertheless, the sequence revealed the presence of valine at 555 amino acid residue instead of isoleucine, which is not a feature of traditional CPV-2a (PARRISH et al., 1991; MARTELLA et al., 2004). Again a new variant of CPV-2a strains was found to possess this mutation at 555 i. e., isoleucine to valine (MARTELLA et al., 2006; PEREIRA et al., 2007; DECARO and BOUNAVOGLIA, 2012). This new CPV-2a variant was also the prominent type in Beijing, China (WANG et al., 2013; WU et al., 2015). This variant has only been previously identified in India in two places, Tamil Nadu and Uttar Pradesh. Following examination of 22 isolates over a period of two years, CHINCHKAR et al. (2006) reported CPV-2a as the predominant antigenic variant prevalent in Southern and Central India. According to a recent report, CPV-2a is a predominant variant in the whole of Asia (DECARO and BUONAVOGLIA, 2012).

The evolutionary analysis revealed that the Indian CVP-2a isolates are of distinct lineage from other countries, such as the USA, China, Italy, Korea and Taiwan. In previous studies the canine parvovirus isolates in India also appeared to have evolved independently and formed separate lineages distinct from the South East Asian isolates (CHINCHKAR et al., 2006). There were two clades separating the South Indian (Tamil Nadu) CPV-2a variant with other Indian isolates (Andhra Pradesh, Maharashtra, Uttar Pradesh, New Delhi and Gujarat). Previous reports suggested that the CPV-2b was predominant in North Indian regions, which are being replaced by the new CPV-2a variant. However, the South Indian isolates were predominant, with CPV-2a infections (NANDI et al., 2013). The new variant was first identified in China (YI et al., 2009; ZHANG et al., 2010) and Thailand (PHROMNOI et al., 2010) and subsequently in a few places in India, such as Tamil Nadu and Uttar Pradesh between 2011 and 2012. The results suggest the North East Asian origin of this new CPV-2a variant and the Indian incidences may be due to the transborder movement of animals. This new CPV-2a variant appeared in around 2004 in China and Korea (WANG et al., 2013; JEOUNG et al., 2008).

Along with the above CPV-2a variant, another new variant (lower band of 525 bp) was obtained in a few cases. The presence of this new variant in all these cases was detected along with the above CPV-2a variant, which suggests that the new variant's presence and co-occurrence is possible only in association with CPV-2a, and not as a separate entity. It might be a completely new variant or a defective viral strain of CPV-2a which needs to be investigated further. The antigenic properties of this new variant, with six extra transitions compared to the CPV-2a variant, needs to be further studied.

Conclusion

In the present study, the fast boiling method seemed to be an easier and economical method of virus screening by PCR from faecal samples. Both blood as well as faecal samples should be screened for virus detection in clinical cases, with appropriate collection of the history of cases on vaccination. The new CPV-2a strain is only prevalent in and around Bhubaneswar, Odisha. A new strain was also found out along with CPV-2a in the form of mixed infections, and needs further characterization. Several non-synonymous mutations found in the new strains demonstrated changes in the virus antigenisity, making molecular epidemiological surveillance important and thus the redesign of the antigenicity vaccine formula.

Conflict of interest statement

The authors are not aware of any conflict of interest associated with this manuscript.

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References

BANJA, B. K., N. SAHOO, H. K. PANDA, S. K. RAY, P. K. DAS (2002): Epizootiological status of canine viral haemorrhagic gastroenteritis in Bhubaneswar city. Indian Vet. J. 79, 850-851.

BATTILANI, M., S. CIULLI, E. TISATO, S. PROSPERI (2002): Genetic analysis of canine parvovirus isolates (CPV-2) from dogs in Italy. Virus Res. 83, 149-157.

BELAK, S., A. BALLAGI-PORDANY (1993): Application of the polymerase chain reaction (PCR) in veterinary diagnostic virology. Vet. Res. Com. 17, 55-72.

BUONAVOGLIA, C., V. MARTELLA, A. PRATELLI, M. TEMPESTA, A. CAVALLI, D. BUONAVOGLIA, G. BOZZO, G. ELIA, N. DECARO, L. E. CARMICHAEL (2001): Evidence for evolution of canine parvovirus type-2 in Italy. J. Gen. Virol. 82, 1555-1560.

CALDERON, M. G, C. ROMANUTTI, M. WILDA, A. D. ANTUONO, L. KELLER, M. N. GIACOMODONATO, N. MATTION, J. L. TORRE (2015): Resurgence of canine parvovirus 2a strain in domestic dog population from Argentina. J. Virol. Methods. 222, 145-149.

CHINCHKAR, S. R., B. M. SUBRAMANIAN, N. H. RAO, P. N. RANGARAJAN, D. THIAGARAJAN, V. A. SRINIVASAN (2006): Analysis of VP2 gene of canine parvovirus in India. Arch. Virol. 151, 1881-1887.

DECARO, N., C. DESARIO, A. PARISI, V. MARTELLA, A. LORUSSO, A. MICCOLUPO, V. MARI, M. L. COLAIANNI, A. CAVALLI, L. DI TRANI, C. BUONAVOGLIA (2009): Genetic analysis of canine parvovirus type 2c. Virology 385, 5-10.

DECARO, N., C. BUONAVOGLIA (2012): Canine parvovirus - a review of epidemiological and diagnostic aspects, with emphasis on type 2c. Vet. Microbiol. 155, 1-12.

JEOUNG, S. Y., S. J. AHN, D. KIM (2008): Genetic analysis of VP2 gene of canine parvovirus isolates in Korea. J. Vet. Med. Sci. 70, 719-722.

JOSHI, D. V., S. P. SINGH, S. P. SHARMA, B. J. PATEL (1997): Scanning electron microscopical features of experimental canine parvoviral enteritis. Indian J. Vet. Pathol. 21, 95-98.

KOCH, W. C., S. P. ADLER (1990): Detection of human parvovirus B19 DNA by using the polymerase chain reaction. J. Clin. Microbiol. 28, 65-69.

KUMAR, M., S. NANDI (2010): Molecular typing of canine parvovirus by polymerase chain reaction and restriction enzyme analysis. Transbound. Emerg. Dis. 57, 458-463.

KUMAR, S., M. NEI, J. DUDLEY, K. TAMURA (2008): MEGA: a biologist centric software for evolutionary analysis of DNA and protein sequences. Brief. Bioinform. 9, 299-306.

MacLACHLAN, N., E. J. DUBOVI (2011): *Parvoviridae.* In: Fenner's Veterinary Virology. 4th ed. Oxford, Elsevier. pp. 225-235.

MARTELLA, V., A. CAVALLI, A. PRATELLI, G. BOZZO, M. CAMERO, D. BUONAVOGLIA, D. NARCISI, A. TEMPESTA, C. BUONAVOGLIA (2004): A canine parvovirus mutant is spreading in Italy. J. Clin. Microbiol. 42, 1333-1336.

MARTELLA, V., N. DECARO, C. BUONAVOGLIA (2006): Evolution of CPV-2 and implication for antigenic/genetic characterization. Virus Genes 33, 11-13.

MIRANDA, C., C. R. PARRISH, G. THOMPSON (2016): Epidemiological evolution of canine parvovirus in the Portuguese domestic dog population. Vet. Microbiol. 183, 37-42.

MITTAL, M., S. CHAKRAVARTI, J. K. MOHAPATRA, P. K. CHUG, R. DUBEY, V. UPMANUYU, P. S. NARWAL, A. KUMAR, C. P. CHURAMANI, N. S. KANWAR (2014): Molecular typing of canine parvovirus strains circulating from 2008 to 2012 in an organized kennel in India reveals the possibility of vaccination failure. Infect. Genet. Evol. 23, 1-6.

MOHAN RAJ, J., H. K. MUKHOPADHYAY, J. THANISLASS, P. X. ANTONY, R. M. PILLAI (2010): Isolation, molecular characterization and phylogenetic analysis of canine parvovirus. Infect. Gen. Evol. 10, 1237-1241.

MOLITOR, T. W., K. ORAVEERAKUL, Q. Q. ZHANG, C. S. CHOI, L. R. LUDEMANN (1991): Polymerase Chain Reaction (PCR) amplification for the detection of porcine parvovirus. J. Virol. Methods 32, 201-211.

MUKHOPADHYAY, H. K., S. L. MATTA, S. AMSAVENI, P. X. ANTONY, J. THANISLASS, R. M. PILLAI (2014): Phylogenetic analysis of canine parvovirus partial VP2 gene in India. Virus Genes 48, 89-95.

MURPHY, F. A., E. P. J. GIBBS, M. C. HORZINEK, M. J. STUDDERT (1999): *Parvoviridae.* In: Veterinary Virology, 3rd ed. Academic Press, San Diego, USA. pp. 343-356.

NANDI, S., M. KUMAR, T. K. MOHAPATRA, C. RAVISHANKAR (2013): Emergence of canine parvovirus-2 variants and its impact on vaccination. World Appl. Sci. J. 23, 1366-1376.

NANDI, S., M. KUMAR (2014): Vaccine failure against canine parvovirus infection in dogs. I. J. C. P. 6, 21-30.

PARRISH, C. R., P. H. O'CONNEL, J. F. EVERMANN, L. E. CARMICHAEL (1985): Natural variation of canine parvovirus. Science 230, 1046-1048.

PARRISH, C. R., C. F. AQUARDO, M. L. STRASSHEIM, J. F. EVERMANN, J. SGRO, H. O. MOHAMMED (1991): Rapid antigenic-type replacement and DNA sequence evolution of canine parvovirus. J. Virol. 65, 6544-6552.

PEREIRA, C. A., E. S. LEAL, E. L. DURIGON (2007): Selective regimen shift and demographic growth increase associated with the emergence of high- fitness variants of canine parvovirus. Infect. Genet. Evol. 7, 399-409.

PEREZ, R., P. BIANCHI, L. CALLEROS, L. FRANCIA, M. HERNANDEZ, L. MAYA, Y. PANZERA, K. SOSA, S. ZOLLER (2012): Recent spreading of a divergent canine parvovirus type 2a (CPV-2a) strain in a CPV-2c homogenous population. Vet. Microbiol. 155, 214-219.

PHROMNOI, S., K. SIRINARUMITR, T. SIRINARUMITR (2010): Sequence analysis of VP2 gene of canine parvovirus isolates in Thailand. Virus Genes 41, 23-29.

PHUKAN, A., D. K. SARMA, D. DEKA, P. K. BORO (2005): Standardization of ELISA for detection of canine parvovirus infection. Indian Vet. J. 82, 355-356.

PHUKAN, A., B. BAISHYA, D. DEKA, P. K. BORO (2010) Prevalence of canine parvovirus infection in Assam. Indian Vet. J. 87, 972-974.

PINTO, L. D., A. F. STRECK, K. R. GONCALVES, C. K. SOUZA, A. O. CORBELLINI, L. G. CORBELLINI, C. W. CANAL (2012): Typing of canine parvovirus strains circulating in Brazil between 2008 and 2010. Virus res. 165, 29-33.

REED, A. P., E. V. JONES, T. J. MILLER (1988): Nucleotide sequence and genome organization of canine parvovirus. J. Virol. 62, 266-276.

SAMBROOK, J., E. F. FRITSCH, T. M. MANIATIS (1989): Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SCHUNCK, B., W. KRAFT, U. TRUYEN (1995): A simple touch-down polymerase chain reaction for the detection of canine parvovirus and feline panleukopenia virus in faeces. J. Virol. Methods 55, 427-433.

TSAO, J., M. S. CHAPMAN, M. AGBANDJE, W. KELLER, K. SMITH, M. LUO, T. J. SMITH, M. G. ROSSMANN, R. W. COMPANS, C. R. PARRISH (2008): The three dimensional structure of canine parvovirus and its functional implications. Science 251, 1456-1464.

UWATOKO, K., M. SUNAIRI, M. NAKAJIMA, K. YAMAMURA (1995): Rapid method utilizing polymerase chain reaction for detection of canine parvovirus in faeces of diarrheic dogs. Vet. Microbiol. 43, 315-323.

WANG, J., L. J. SHI, P. WANG, Z. Z. ZHAO, M. M. GONG, G. LI, W. F. YUAN, H. F. ZHU (2013): Genetic characterization of canine parvovirus isolates from Beijing of China between 2009 and 2011. Agric. J. 8, 60-65.

WANG, J., L. LIU, R. LI, J. WANG, Q. FU, W. YUAN (2016): Rapid and sensitive detection of canine parvovirus type 2 by recombinase polymerase amplification. Arch. Virol. DOI 10. 1007/s00705-015-2738-y.

WANG, J., P. LIN, H. ZHAO, Y. CHENG, Z. JIANG, H. ZHU, H. WU, S. CHENG (2016a): Continuing evolution of canine parvovirus in China: Isolation of novel variants with an Ala5Gly mutation in the VP2 protein. Infect. Genet. Evol. 38, 73-78.

WU, J., X. T. GAO, S. H. HOU, X. Y. GUO, X. S. YANG, W. F. YUAN, T. XIN, H. F. ZHU, H. JIA (2015): Molecular epidemiological and phylogenetic analyses of canine parvovirus in domestic dogs and cats in Beijing, 2010-2013. J. Vet. Med. Sci. 77, 1305-1310.

YI, L., S. P. CHENG, X. J. YAN, J. K. WANG, B. LUO (2009): Genetic variation analysis of canine parvovirus VP2 gene in China. Bing. Du. Xue. Bao. 25, 452-459.

ZHANG, Q., X. M. XU, G. Q. ZHAI, Z. WANG, S. H. HOU, H. F. ZHU (2010): Molecular characterization of canine parvovirus strains circulating in China. Afr. J. Biotechnol. 9, 4556-4560.

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SAŽETAK

Pasji parvovirus tip 2 (PPV-2) ima tri glavne antigenske varijante: PPV-2a, PPV-2b i PPV-2c. Radi razumijevanja njegove evolucije i razvijanja učinkovitih mjera suzbijanja potrebno je istražiti njegove mutante iz različitih geografskih područja. U ovom je radu lančanom reakcijom polimerazom bio pretražen 71 uzorak fecesa i 12 uzoraka krvi pasa sa sumnjom na parvovirusnu infekciju u državi Odisha u Indiji. Postupak dobivanja fekalnog lizata brzim ključanjem pri 100 °C pokazao se osjetljivijim u odnosu na ekstrakciju DNA iz uzoraka fecesa fenol-kloroformom. Rezultati su pokazali da je od 71 pretraženog uzorka fecesa 29 bilo pozitivnih (583 bp umnožak), a od 12 pretraženih uzoraka krvi sedam pozitivnih s različitim nalazima kod istih slučajeva, što govori da je za postavljanje dijagnoze potrebno pretražiti oba uzorka od iste životinje. Cijepanje odsječka 583 bp restrikcijskim enzimom *Mbo*II (PCR-RFLP) pokazalo je da izdvojeni soj ne pripada PPV-2c. Daljnjim sekvencioniranjem fragmenata od 583 bp pokazalo se da izolat pripada mutiranoj varijanti PPV-2a. Zanimljivo je da je u 11 pozitivnih uzoraka fecesa usporedno s fragmentima od 583 bp bila dokazana i prisutnost mutanta PPV-2a s fragmentom od 525 bp što iziskuje daljnju karakterizaciju. Te dvije varijante PPV-2a svrstane su u zajedničku skupinu u filogenetskom stablu, različitu od PPV-2b i PPV-2c. Naši rezultati potvrđuju dinamiku promjena varijanata PPV s naglaskom na važnost istraživanja za razumijevanje njegove epizootiologije.

Ključne riječ: pasji parvovirus, dijagnoza, lančana reakcija polimerazom, nova varijanta