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Molecular detection of 2c variant of *Carnivore protoparvovirus1* from Mizoram, India

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Abstract
Canine parvovirus2 (CPV2) is associated with acute fatal hemorrhagic diarrhea in pups. The present study was conducted to detect the variant of *Carnivore protoparvovirus 1*from a case of severe hemorrhagic gastroenteritis in a pup by polymerase chain reaction (PCR) combining with partial *VP2* gene sequencing. PCR was performed, targeting a partial segment of the *VP2* protein gene, which gives an amplicon of 536 bp. PCR amplicon was cloned and sequenced. Phylogenetic analysis was performed by comparing sequences with all other variants of CPV2 viruses, revealed that the virus belonged to the CPV2c variant (MN218607). The partial *VP2* gene sequence revealed a 99.4 to 99.6% sequence similarity with other reported CPV2c variant. Typical substitution of Glutamine (E) instead of Asparagine (N) in the amino acid position 426 could be observed, further confirming the CPV2c variant. Till now, there is no published report available that show the presence of CPV 2c variant in the dog population of Mizoram.

Keywords: *Carnivore protoparvovirus1*, CPV 2c, PCR, Partial *VP2*, Sequence analysis

Introduction
Canine parvovirus (CPV) causes severe gastroenteritis in unvaccinated puppies or puppies with poor maternal protection [1]. The disease was first observed during the early 1970s worldwide and the virus was isolated in both canine and feline cell culture [2]. CPV belongs to the species *Carnivore protoparvovirus 1* of the genus *Protoparvovirus*of subfamily *Parvovirinae*within the family *Parvoviridae*under the order *Piccovirales* [3]. CPV is a variant of the *Feline panleukopenia virus* (FPV) with the new nomenclature of *Canine parvovirus type 2* (CPV 2) and differs genetically and antigenically from the canine minute virus, designated as CPV 1, which also causes neonatal death in dogs [4]. The genome is a linear single-stranded DNA molecule of around 5.2 kb in length covered by an icosahedral capsid having a diameter of around 26 nm [4]. Genome is having two open reading frames (*ORF1* and *ORF2*) where *ORF1* encodes two non-structural proteins (NS1 and NS2) and *ORF2* encodes two structural proteins (VP1 and VP2). There is the presence of another protein VP3 which is produced by proteolytic processing of VP2 [2]. Both the structural proteins assemble the capsid with 54 units of VP1 and 6 units of VP2 [1]. *VP2* was found to play an important role in determining antigenicity and host range of CPV [5]. NS1 and NS2 were found to be essential for viral replication, DNA packaging, cytotoxicity, and pathogenicity and sequence analysis of these non-structural proteins is important for molecular epidemiology investigations [6]. Three variants of CPV2 (CPV2a, CPV2b, and CPV2c) have been reported from clinically infected dogs and cats in different countries and they are differentiated based on phylogenetic analysis and amino acid residues within the VP2 protein [7,8]. Changes in the nucleotide sequence were found to occur at a rate of $1\text{-}10^{-4}$ to $4\text{-}10^{-4}$ changes/nt/year [4]. Although commercial vaccines currently available are mostly based on CPV 2 or CPV 2b, and it is claimed that they can cross-protect against all antigenically distinct types, but the CPV infections are still at large which suggest regular monitoring as an important tool to identify the types of CPV variants related to the infection [9]. Keeping the above points in view, the present study was conducted to detect the variant of *Carnivore protoparvovirus 1* present in Mizoram and molecular characterization of the detected variant based on partial *VP2* gene.

Materials and Methods

Sample collection

Sterile rectal swab sample from a 3 months old puppy with a history of vomition and diarrhea was collected from the Teaching Veterinary Clinical Complex (TVCC) of College of Veterinary Sciences and Animal Husbandry, Aizawl. Virus materials were harvested and stored at -80°C.

DNA extraction

Viral DNA from the sample was extracted by using the QIAamp DNA Mini Kit (Cat. No. 51304, QIAGEN) according to the manufacturer's protocol.

Molecular detection of partial *VP2* gene

The virus DNA was detected by Polymerase Chain Reaction (PCR) by using oligonucleotide primers targeting partial *VP2* gene. Primers used in this studyarecpvVP2-F (5′-CAGGAAGATATCCAGAAGGA-3′) and cpvVP2-R (5′-GGTGCTAGTTGA TATGTAATAAACA-3′) [10]. A total of 25 µl reaction volume was prepared with 2X Green Taq Master Mix (12.5 µl), forward and reverse primers @ 10pmol each, extracted DNA template 2 µl, and volume adjusted with nuclease-free water. The thermal cycler conditions were initial denaturation at 94˚C for 2 min and 30 cycles of denaturation at 94˚C for 30 sec, annealing at 46.3˚C for 30 sec, and extension at 72˚C for 30 sec. A final extension period of 10 min at 72˚C was used for cloning purposes. The amplified PCR product was analyzed on 1.5% agarose gel.

Cloning and sequencing of partial *VP2* gene

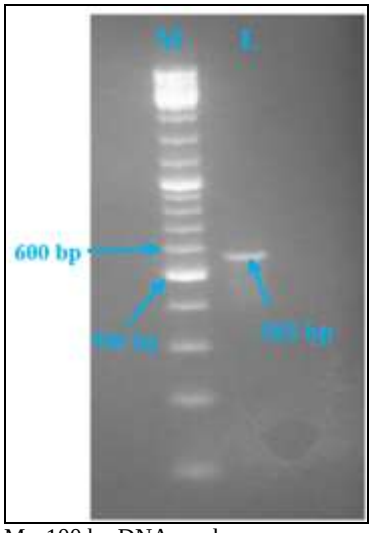
The cloning was performed by usingInsTAclone PCR Cloning Kit (Cat. No. K1213, Thermo Scientific) according to the manufacturer's protocol. One of the confirmed positive clones was sequenced by outsourcing. The sequence was submitted to GenBank for accession number.

Sequence analysis

The sequence analysis was carried out using multiple alignment program of MEGA9 software. The phylogenetic tree was generated by using the Neighbor-Joining method, keeping bootstrap consensus from 1000 replicates. For comparison, sequence data representing all the genetic variants of CPV2 including the vaccine strain of the virus was considered for generating the tree.

Results and Discussion

The harvested virus material from the clinically affected pup gives the desired PCR amplification of approximately 583 bp fragments of the *VP2* and *VP3*genes (Figure 1). Cloning was performed and confirmed by colony PCR and subsequently sequenced by outsourcing. The sequence was annotated and submitted to GenBank. Phylogenetic tree analysis based on partial *VP2* (536 bp) sequence of the present isolate (MN218607) shows a close cluster with other 2c strains as compared to the FPV, CPV 2, 2a, new 2a, 2b, and new 2b strains (Figure 2). The present isolate has clustered closely with the 2c strain of Zambia (LC409283) as compared with other 2c strains from India (KX425920) and China (MF001435, MH685923). Nucleotide homology of the present isolate is ranging between 99.4-99.6% similarity when compared with other 2c sequences (MK517994, MH685923, MF001435, LC409283). However, when compared with the CPV2b sequence (KX469430, KX425921) the present isolate is showing 99.1% homology. A homology study with CPV2a (KR703272, KU866417) sequences revealed that the similarity ranges between 97.9 to 98.3%. Again, sequence comparison with vaccine strains (JN625224 and KP406926) revealed a homology of 98.1%. Thus the analysis established the present isolate as CPV2c variant. The strain has two more substitutions at position 1432 (T→C) is matching with Zambia strain (LC409283) and at position 1503 (A→G) is similar to China strain (MK517994). There are four nucleotide variations with the India 2c strain (KX425920) at 1320 (A→T), 1432 (T→C), 1503 (A→G), and 1659 (T→C). Deduced amino acid sequence analysis of partial VP2 protein shows the typical substitution of Asparagine (N) by Glutamine (E) at the position of 426 of CPV 2c sequence, which is considered to be a signature tag of CPV 2c strain (Table 1). In an evolutionary study of CPV 2c from Taiwan in 2016, it was reported 98.5 to 100% similarity within CPV2c strains and 97.7 to 100% homology with CPV sequences reported from Italy and China. *VP2* sequence within the variants exhibits a close homology which ranged from 99 to 100% and slight diversity among CPV2a and CPV2b variants within a range of 96.9 to 99.2% similarity [11-13]. The characteristic substitution at position 426 (N→E) has been reported from all the CPV2c strain in India and abroad [14-17]. Phylogenetic analysis and deduced amino acid sequence comparison of partial *VP2* gene revealed the involvement of CPV2c strain in the case of canine parvoviral diarrhea in Mizoram. This is the first report of the presence of CPV2c from North-East India (Mizoram) with a mutation at position 1320 (A→T).



M - 100 bp DNA marker
L - Positive amplicon of partial *VP2* and *VP3* genes (583 bp)

Fig 1: PCR amplification of partial *VP2* and *VP3* genes of CPV 2 virus observed in 1.5% agarose gel

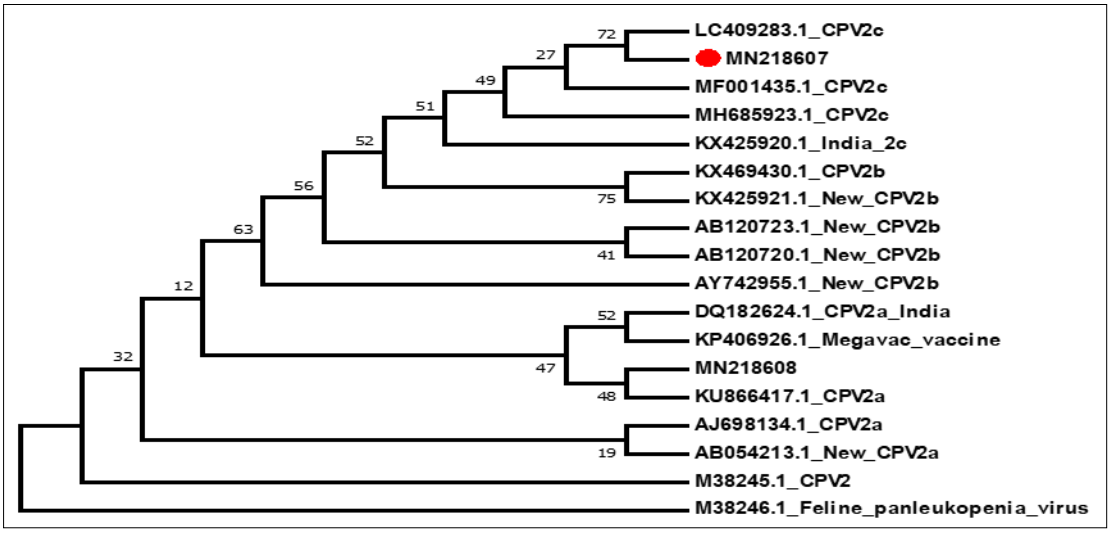


Fig 2: Phylogenetic tree based on partial *VP2* gene sequence (536 bp) by neighbour-joining method (MEGA9) showing a close cluster of the present strain (MN218607) with other CPV 2c variants reported from India and abroad.

Table 1: Amino acid substitution of VP2 of CPV variants along with FPV and Megavac vaccine

Variant	Accession no.	Amino acid position of VP2			
		426	466	564	568
FPV	M38246	Asn (N)	Asn (N)	Asn (N)	Ala (A)
CPV 2	M38245	Asn (N)	Asn (N)	Ser (S)	Gly (G)
Megavac	KP406926	Asn (N)	Asp (D)	Ser (S)	Gly (G)
CPV 2a	DQ182624	Asn (N)	Asn (N)	Ser (S)	Gly (G)
CPV 2a new	AB054213	Asn (N)	Asn (N)	Ser (S)	Gly (G)
CPV 2b	KX469430	Asp (D)	Asn (N)	Ser (S)	Gly (G)
CPV 2b new	KX425921	Asp (D)	Asn (N)	Ser (S)	Gly (G)
CPV 2c	KX425920	Glu (E)	Asn (N)	Ser (S)	Gly (G)
	MF001435	Glu (E)	Asn (N)	Ser (S)	Gly (G)
	MH685923	Glu (E)	Asn (N)	Ser (S)	Gly (G)
	MN218607 (Present study)	Glu (E)	Asn (N)	Ser (S)	Gly (G)

Conclusion

In the present study, we had detected the 2c variant of *Carnivore protoparvovirus 1* using PCR based on the amplification of partial *VP2* gene which was confirmed as a 2c variant by sequence analysis. To date, there is no other published report available that shows the presence of a 2c variant of *Carnivore protoparvovirus 1* in the dog population of Mizoram, India.

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