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Molecular Characterization and Phylogenetic Analysis of Canine Parvovirus Type 2 (CPV-2) in the Aizawl Region of Mizoram Reveals Circulation of CPV-2c Antigenic Variant: A Hospital based Study

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ABSTRACT

Background: The objective of the present study was to confirm the presence of CPV-2 in dogs with acute gastroenteritis and to characterize the antigenic variants circulating in the Aizawl region of Mizoram, India.

Methods: A hospital based epidemiological study was carried out from April, 2019 to January, 2020. Faecal samples were collected from the dogs with gastroenteritis and screened for CPV-2 infection by polymerase chain reaction (PCR). The distribution of positive cases according to various epidemiological factors was studied. Partial sequencing of VP2 gene was performed for the analysis of CPV-2 variants.

Result: A total of 96 faecal samples were collected during the study period out of which 70 (72.91%) were found positive for CPV-2 infection. The distribution of positive cases was highest in young dogs of the age group between 3-6 months (47.14%), cross-bred (48.57%) and unvaccinated dogs (64.28%). The percentage of CPV-2 infection was relatively higher in male (54.28%) as compared to female dogs (45.71%). The analysis of nucleotide sequences from amplified products and phylogenetic analysis revealed presence of CPV-2a (1/5, 20.0%) and CPV-2c (4/5, 80.0%). Furthermore, sequence analysis of large number of samples is required to ensure the prevalent antigenic variant of CPV-2 linked to the infection.

Key words: CPV-2c, Dogs, Mizoram, PCR, VP2 gene.

INTRODUCTION

Canine parvovirus (CPV), one of the most common enteric viral pathogens of domestic dogs, causes acute gastroenteritis leading to high morbidity and mortality throughout the world (Neeraj *et al.,* 2020; Eregowda *et al.,* 2020). CPV, a *Carnivore Protoparvovirus 1* virus of the genus *Protoparvovirus* of subfamily *Parvovirinae* within the family *Parvoviridae* under the order *Piccovirales*, is a non-enveloped, single-stranded DNA virus (Akter *et al.,* 2020; Kwan *et al.,* 2021). When CPV emerged in 1978 as a new virus infecting dogs it caused a severe disease amongst domestic and wild dogs with clinical signs including fever, vomiting and haemorrhagic diarrhoea, as well as myocarditis in young puppies (Decaro and Buonavoglia, 2012). The virus was named canine parvovirus type 2 (CPV-2) to distinguish it from canine parvovirus type 1 (CPV-1), commonly known as canine minute virus (Kwan *et al.,* 2021). CPV-2 probably arose from a very closely related virus in cats, the feline panleukopenia virus (FPV) through a small number of mutations in the single capsid protein (Thomas *et al.,* 2014). Within a few years, original CPV-2 was replaced in the field by new antigenic variants such as CPV-2a, CPV-2b and CPV-2c (Thomas *et al.,* 2017; Gaykwad *et al.,* 2018). The CPV-2 genome is 5.2 kb long containing two major open reading frames (ORFs), ORF1 and ORF2, which encode non-structural proteins NS1 and NS2 and capsid proteins VP1 and VP2, respectively (Castillo *et al.,* 2020). The antigenic variants of CPV-2 are classified based on the amino acid present at position 426 of the VP2 capsid protein, asparagine (Asn) for CPV-2a, aspartic acid (Asp) for CPV-2b and glutamic acid (Glu) for CPV-2c (Decaro and Buonavoglia, 2012). In recent times, new antigenic variants of CPV-2a/b emerged, generated by a specific mutation at position 297 [Serine-Alanine (Ser-Ala)], which has been designated as new CPV-2a/b variants (Castillo *et al.,* 2020).

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Mizoram is one of the eight north-eastern (NE) states of India. The region's climate is humid-tropical, with short winters, long summers and heavy rainfall. The present study was undertaken to confirm the presence of CPV-2 in dogs with acute gastroenteritis and to characterize the antigenic variants circulating in the Aizawl region of Mizoram, India.

MATERIALS AND METHODS

Selection of animals and collection of faecal samples

A hospital based study was conducted at Teaching Veterinary Clinical Complex (TVCC), College of Veterinary Sciences and Animal Husbandry (CVSc and AH), Central Agricultural University (CAU), Aizawl, Mizoram, India. Dogs with a history of weakness, reduced appetite, diarrhea/ hemorrhagic diarrhea, vomition, dehydration with or without pyrexia were included in the present study. The faecal samples from diarrheic dogs were collected in a sterile swab and transported on ice to laboratory. A total of 96 faecal samples were collected from April, 2019 to January, 2020. The epidemiological parameters like age, breed, sex and vaccination status were recorded from the affected dogs. The experimental protocol (No.F.25/17/2019-CPCSEA) was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals, India.

Detection of CPV-2 by polymerase chain reaction

The screening of CPV-2 in faecal samples was carried out by direct detection of viral genome using polymerase chain reaction (PCR) targeting partial VP2 gene. The faecal samples collected in a sterile swab were immersed immediately in a tube containing phosphate buffer saline (1×PBS; pH 7.3±0.1) and stored at –20°C until further analysis. The faecal samples were centrifuged at 8,000 rpm for 5 min at 4°C and 200 µL supernatant was taken and processed for DNA extraction. Total genomic DNA from all the collected faecal samples was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. The diagnosis of CPV-2 infection was carried out by using PCR primers [*555for* 5′CAGGAAGATATCCAGAAGGA3′ (from 4003 to 4022) and *555rev* 5′GGTGCTAGTTGATATGTAATAAACA3′ (from 4585 to 4561)] which gives an amplicon size of 583 bp out of which it covers 536 bp fragment of VP2 towards 5′ end (Buonavoglia *et al.,* 2001). The PCR-amplified products were resolved on 1.5% agarose gel in Tris–acetate–EDTA (TAE) buffer. The agarose gel was visualized under ultraviolet (UV) light in a gel documentation system (Molecular Imager® Gel Doc™ XR+ System, Bio-Rad, USA).

Cloning and partial sequencing of the VP2 gene

The PCR amplified partial fragment of the VP2 gene from five faecal samples were cloned by using InsTAclone PCR Cloning Kit (Cat. No. K1213, Thermo Scientific) as per the manufacturer's protocol. The confirmed clones were sent to the University of Delhi, South Campus, New Delhi, for sequencing. Sequencing results were annotated and submitted to Genbank, National Center for Biotechnology Information (NCBI) to get the accession numbers.

Phylogenetic analysis

Phylogenetic analysis based on partial sequencing of the VP2 gene of CPV-2 variants was performed by sequence comparison with other sequences retrieved from the database (NCBI). Sixteen published sequences comprising different variants of CPV-2 including vaccine strain and FPV were used to generate phylogenetic tree. Alignment of the nucleotide sequences was performed by ClustalW multiple alignment method. Phylogenetic analysis was performed by Maximum Likelihood method of MEGA (Molecular Evolutionary Genetics Analysis) software (version 9). Bootstrap values were calculated based on 1,000 replicates. Percent identity of the aligned sequences was calculated using Mega align of DNASTAR (version 6). Sequence chromatogram was visualized by BioEdit v 7.0.5 analysis software (Isis Therapeutics, Carlsbad, CA, USA).

Statistical analysis

The data were structured in the Microsoft Excel spreadsheet. Descriptive statistics were performed and reported as percent positivity of infections.

RESULTS AND DISCUSSION

Detection of CPV-2 virus in dogs with gastroenteritis

During the study period, a total of 96 faecal samples were collected from the dogs with gastroenteritis. The diagnosis of CPV-2 infection was carried out by PCR. Out of 96 samples, 70 (72.91%) were found positive for CPV-2 infection as evident by the presence of 583 bp amplicon size (Fig 1). The primary causes of viral gastroenteritis in dogs include CPV-2, canine coronavirus, canine rotavirus and canine distemper virus. CPV-2 infection causes acute gastroenteritis characterized by vomiting and blood mixed loose stools, anorexia, depression, lethargy and fever (Chethan *et al.,* 2016). Most infectious and non-infectious causes of gastroenteritis produce clinical signs that are overlapping in pattern, making it challenging to diagnose CPV-2 infection merely based on clinical signs (Chethan *et al.,* 2021). The PCR technique is widely used to diagnose CPV-2 infection as it provides a rapid, sensitive and accurate diagnosis (Thomas *et al.,* 2014). The high percentage of CPV-2 infection in suspected gastroenteritis cases might be due to easy transmission of infection via faecal-oral route, resistant nature of the virus to routinely used disinfectants and persistence of virus in the environment for prolonged periods in subtropical humid climatic conditions (Chethan *et al.,* 2021). CPV-2 is ubiquitous and can survive for more than a year in the environment, allowing susceptible dogs to be exposed to infected faeces, vomitus, or fomites (Mylonakis *et al.,* 2016; Geetha and Selvaraju, 2021).

Analysis of CPV-2 positive samples based on age, breed, sex and vaccination status

The distribution of positive cases was highest in young dogs of the age group 3-6 months (47.14%) and lowest in >1 year aged dogs (1.42%) (Fig 2). CPV-2 commonly affects

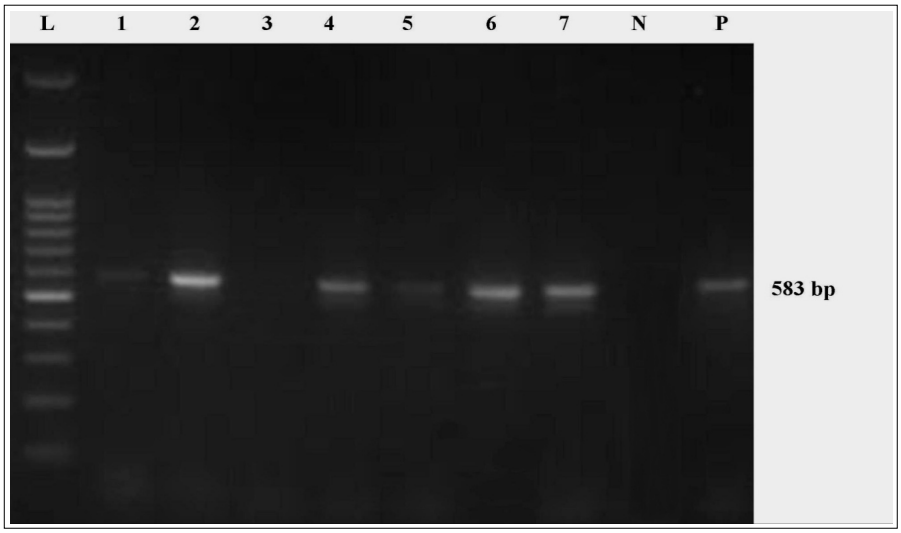

Fig 1: 1.5% agarose gel electrophoresis of the PCR products for identification of CPV-2 in faecal samples collected from dogs with gastroenteritis. Lane L denotes 100 bp DNA ladder, lane P denotes positive control (product size 583 bp), lane N denotes negative control, lanes 1, 2 and 4-7 denote positive blood samples and lane 3 denotes negative sample.

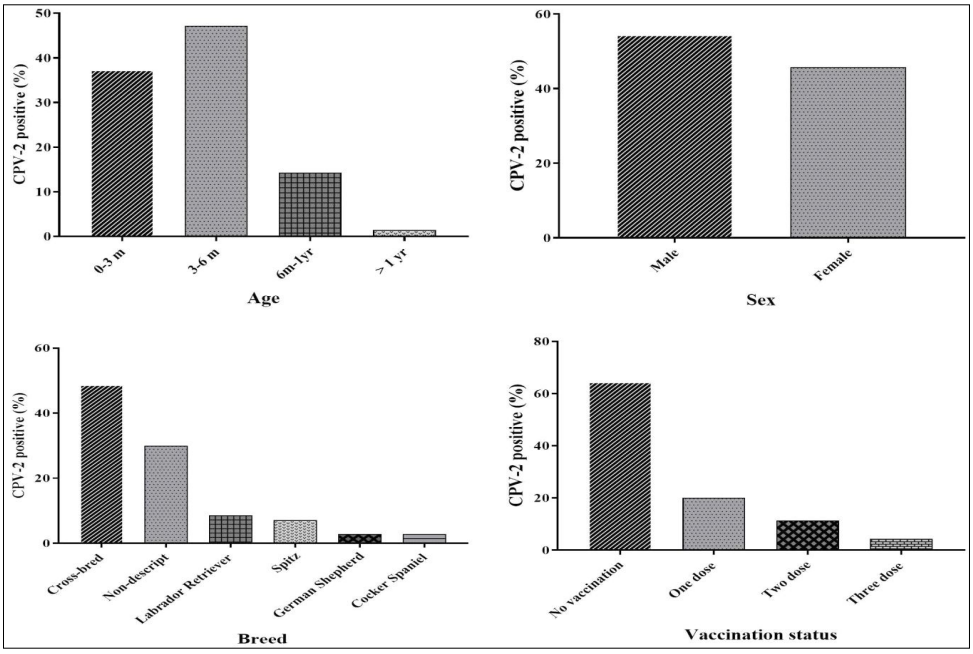

Fig 2: Analysis of CPV-2 positive samples based on different epidemiological parameters.

young puppies between 6 and 20 weeks of age, but unvaccinated dogs of any age group may potentially be affected (Eregowda *et al.,* 2020). Maternally derived antibodies protect newborn puppies from CPV-2 infection; however susceptibility increases when maternally acquired antibodies begin to wane (Chethan *et al.,* 2021). CPV-2 mainly replicates in rapidly dividing cells (myocardiocytes, small intestinal crypt epithelial cells, lymphopoietic tissue and bone marrow), young animals will have a greater number of such cells than adults (Bhanuprakash *et al.,* 2015; Chethan *et al.,* 2021). The present finding revealed that the disease was more common in cross-bred (48.57%) dogs (Fig 2). The percentage of CPV-2 infection was relatively higher in male (54.28%) as compared to female dogs (45.71%) (Fig 2), which could be attributable to the fact that the number of male animals presented to the hospital was higher than female animals. There is no breed or sex susceptibility to CPV-2 infection (Chethan *et al.,* 2021). Breed predisposition of CPV-2 infection is subjected to considerable geographic variation (Mylonakis *et al.,* 2016). When compared to other breeds, the majority of dogs presented to the hospital in the current study were cross-bred dogs. This may be the reason for relatively high percentage of positive cases observed in cross-bred dogs.

The percentage of CPV-2 infection was found to be highest in unvaccinated dogs (64.28%) and lowest in dogs that received three doses of vaccine (4.28%) (Fig 2). Vaccination is the most efficient strategy for the control of CPV-2 infection (Thomas *et al.,* 2014). Modified live vaccines (MLVs) are being used worldwide to provide extended protection against the disease as well as infection. Three doses of vaccines are recommended, with the first dose given at 6-8 weeks of age and then two booster doses every 2-4 weeks until 16 weeks of age or older (Day *et al.,* 2016). Infection in immunized dogs is most likely owing to insufficient antibody production in the presence of high levels of persistent maternal antibodies, or variations in viral genetic make-up between vaccine strain and field strain (Thomas *et al.,* 2014; Chethan *et al.,* 2021). The 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 widespread, suggesting regular monitoring as an important tool to identify the types of CPV variants linked to the infection (Akter *et al.,* 2020). There are also some concerns about the efficacy of CPV-2 based vaccines against new antigenic variants (Thomas *et al.,* 2014).

Molecular characterization and phylogenetic analysis of CPV-2 variants

CPV-2 constantly changes its genetic and antigenic character through regular mutations in the VP2 gene and the resulting antigenic variants (2a, 2b and 2c) are spreading throughout the world (Thomas *et al.,* 2017). Estimates suggest that the changes in the nucleotide sequence were found to occur at a rate of 1×10^{-4} to 4×10^{-4} changes/nt/year (Buonavoglia *et al.,* 2001). It has been reported that CPV-2a is the major antigenic variant of CPV-2 present in India, followed by CPV-2b (Thomas *et al.,* 2017). Out of 70 PCR positive samples, 5 samples were randomly selected for further molecular characterization and phylogenetic analysis. The genotype of CPV-2 variants in this study was determined based on phylogenetic analysis and key amino acid residues of VP2 gene (536 bp). Four isolates (MN909743, MN909745, MN909746 and MN909747) (4/5, 80.0%) were found to be in a compact cluster with CPV-2c sequences, whereas one isolate (MN909744) (1/5, 20.0%) was found to be in the same clad with CPV-2a sequences (Fig 3). 99.6-99.8% sequence identity could be observed based on a partial length sequence of VP2 gene of CPV-2c isolates obtained in the present study. All the four CPV-2c sequences had a sequence similarity ranged between 99.4-99.8% when compared with other CPV-2c sequences retrieved from the database. CPV-2a isolate of the present study had a sequence similarity ranged between 98.9-99.6% when compared with other CPV-2a sequences (Table 1). Deduced amino acid sequence analysis of CPV-2c isolates showed the presence of Glu instead of Asn (CPV-2, 2a, new 2a) and Asp (2b, new 2b) at position 426 of the VP2 capsid

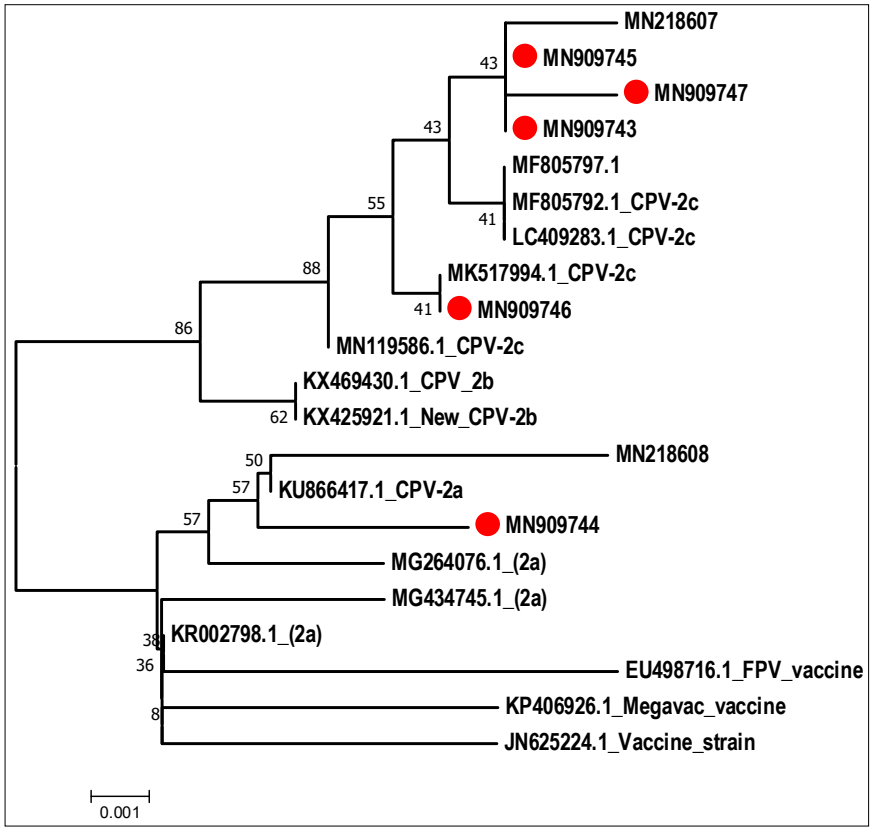

Fig 3: A phylogenetic tree based on five partial VP2 gene sequences from the present study and sixteen published sequences including vaccine strains and FPV.

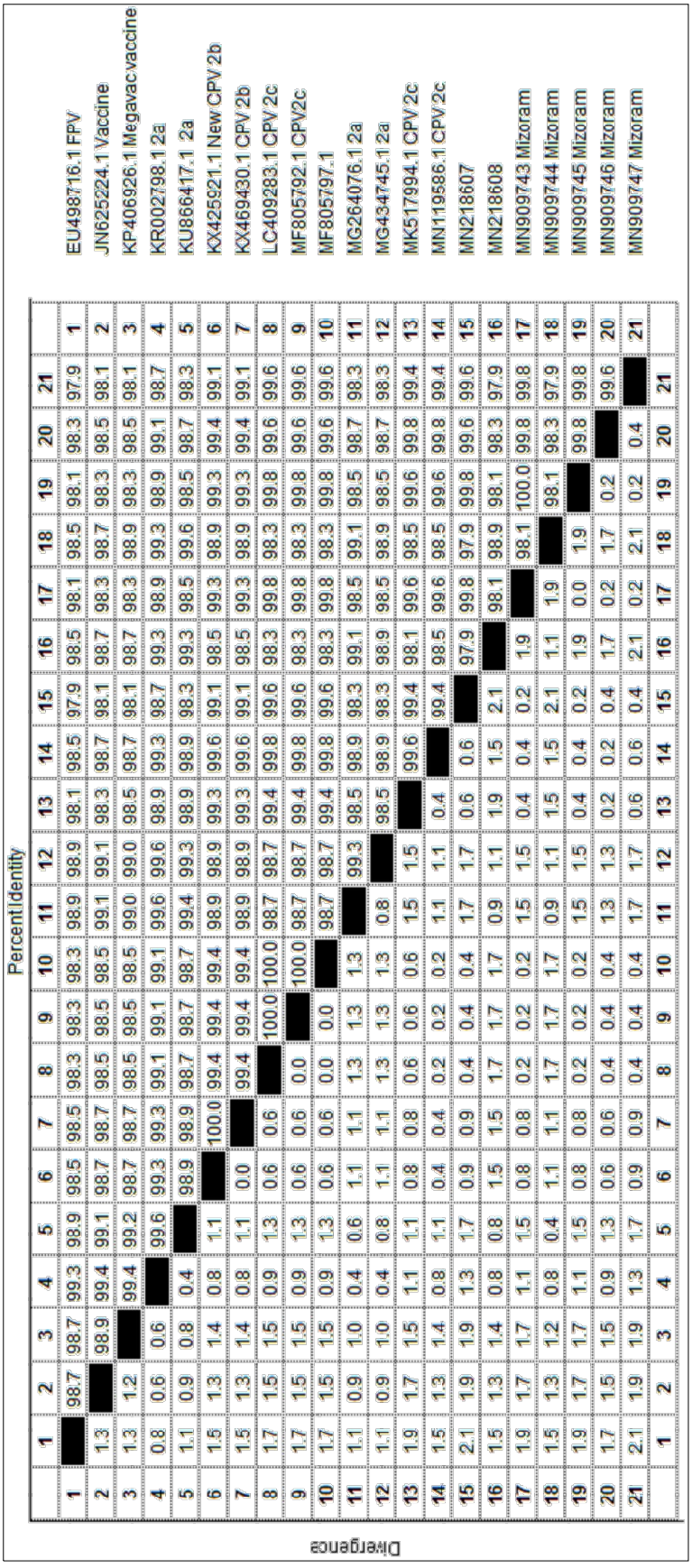

Table 1: Percent identity of VP2 gene (partial sequence) of CPV-2 isolates compared with published reference sequences.

Table 2: Amino acid substitution of VP2 of CPV-2 variants along with vaccine strain and FPV.

Variant	Accession no.	Amino acid position of VP2		
		426	564	568
CPV-2	M38245	Asn (N)	Ser (S)	Gly (G)
Megavac	KP406926	Asn (N)	Ser (S)	Gly (G)
FPV	EU498716	Asn (N)	Asn (N)	Ala (A)
Present study (CPV-2a)	MN909744	Asn (N)	Ser (S)	Gly (G)
CPV-2a	MG264076	Asn (N)	Ser (S)	Gly (G)
New CPV-2a	AB054213	Asn (N)	Ser (S)	Gly (G)
CPV-2b	KX469430	Asp (D)	Ser (S)	Gly (G)
New CPV-2b	KX425921	Asp (D)	Ser (S)	Gly (G)
Present study (CPV-2c)	MN909743	Glu (E)	Ser (S)	Gly (G)
Present study (CPV-2c)	MN909745	Glu (E)	Ser (S)	Gly (G)
Present study (CPV-2c)	MN909746	Glu (E)	Ser (S)	Gly (G)
Present study (CPV-2c)	MN909747	Glu (E)	Ser (S)	Gly (G)
CPV-2c	LC409283	Glu (E)	Ser (S)	Gly (G)

Ala (A): Alanine, Asn (N): Asparagine, Asp (D): Aspartic acid, Glu (E): Glutamic acid, Gly (G): Glycine, Ser (S): Serine.

protein, which is considered to be a signature tag of the CPV-2c antigenic variant (Table 2) (Decaro and Buonavoglia, 2012; Akter *et al.,* 2020; Castillo *et al.,* 2020). Although the sample size in the present study is less, it is important to note that CPV-2c mutants have been evolved to emerge as pathogens of dogs in India. The occurrence of CPV-2c antigenic variant has been reported from northern and southern parts of India (Nandi *et al.,* 2010; Surendhar *et al.,* 2019).

CONCLUSION

In the present study, 72.91% of total gastroenteritis cases were confirmed to be positive for CPV-2 infection. The presence of CPV-2c antigenic variant in the Aizawl region of Mizoram, India supports the notion that CPV-2c is spreading globally and provides new information to understand the evolution of CPV-2 antigenic variants. Furthermore, sequence analysis of large number of samples is required to ensure the prevalent antigenic variant of CPV-2 linked to the infection in the present region.

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Conflict of interest

The authors declare that they have no conflict of interest.

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