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Original Article

The first comprehensive study on isolation and genetic characterization of canine parvoviruses from dogs in Mizoram, India reveals the emergence of CPV-2c

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Abstract

Background: Canine parvovirus type-2 (CPV-2) is a highly contagious enteric pathogen of puppies with worldwide distribution. **Aims:** Molecular epidemiology, genetic characterization, phylogenetic analysis, and isolation of the CPV-2 virus from clinically affected dogs in Mizoram, India over eight years. **Methods:** A total of 202 samples (199 fecal samples, 2 vomita, and 1 tissue sample) were screened by PCR assay. **Results:** 103 out of 202 samples (50.99%) tested positive. Of the 103 positive samples, 83 samples were cloned and sequenced. Sequence analysis showed CPV-2c as the predominant variant (63.85%) followed by the 2a variant (26.5%), 2b (8.43%), and FPV (1.2%). Phylogenetic analyses of the CPV-2c sequences formed separate clusters and were ancestrally related to Japanese, Chinese, and Italian 2c sequences. Similarly, 2a isolates formed separate clusters under different clades and were ancestrally related to Indian, Singaporean, Japanese, Uruguayan, and Chinese 2a isolates. 2b isolates formed a single cluster with the Chinese 2b isolate. FPV isolate clustered with North American FPV. Both synonymous and non-synonymous mutations (unique to this study) were evident in all the types of CPV-2s indicative of active evolution with regional variation. In the cell culture medium, CPV-2 showed cytopathogenic effects at the third passage level. **Conclusion:** The study, the first in-depth report on CPV-2, showed a shift towards CPV-2c as the predominant variant in Mizoram. This variant clustered separately from current vaccine strains, highlighting the need for extensive epidemiological surveillance to better understand viral phylogenomics and evaluate current vaccine efficacy.

Key words: CPV-2c, Epidemiology, Isolation, Mizoram, Mutations

Introduction

Canine parvovirus type-2 (CPV-2) is a highly contagious enteric pathogen of puppies that causes fatal hemorrhagic gastroenteritis. It belongs to the species *Protoparvovirus carnivoran 1*, genus *Protoparvovirus*, and family *Parvoviridae* (Cotmore *et al*., 2019). The original CPV-2 was first identified as a canine host range variant of feline panleucopenia virus (FPV) in 1978 (Zhou *et al*., 2017). In the 1980s, the original CPV-2 underwent substitutional mutations of several *VP2* amino acid residues, including M87L, G300A, Y305N, and

V555I to give rise to CPV-2a (Parrish *et al*., 1991). In 1984, an additional substitution mutation at *VP2*-426 amino acid residue to aspartate (D) engendered CPV-2b (Parrish *et al*., 1991). In 2000, CPV-2c was reported in Italy which had a further substitution mutation as Asp426Glu (Buonavoglia *et al*., 2001). Sequence analysis of recent CPV-2a isolates has revealed a reversion at position 555 to the sequence of FPV/CPV-2, Ile to Val. This mutation restricts the differences among the antigenic variants CPV-2a, 2b, and 2c to only one amino acid at position 426, which are Asn in CPV-2a, Asp in CPV-2b, and Glu in the CPV-2c (Decaro *et al*., 2020). Additionally, new CPV-2a and -2b variants have been identified by alteration of the VP2 amino acid sequence from serine (S) to alanine (A) at amino acid residue 297. Asp-300 2a/2b (Asp in place of Gly at amino acid residue 300) CPVs are regarded as mutants of CPV-2a (Asn at amino acid residue 426) and CPV-2b (Asp at amino acid residue 426) (Decaro and Buonavoglia, 2012; Decaro *et al*., 2020). All these variants have completely replaced the original antigenic type (CPV-2). Despite the intensive vaccination programs, the infection still causes significant fatality in puppies due to multiple variants of the CPV-2 virus, vaccination of non-responders, and strain variations between the vaccine and circulating virus (Decaro *et al*., 2020; Qi *et al*., 2020).

Epidemiological surveys in different countries have shown that CPV-2a is the predominant variant in most Asian and European countries. The CPV-2b variant was found to be the predominant antigenic variant in Ireland, the UK, the U.S.A., African countries, Asian countries, and Australia (Zhou *et al*., 2017; Clark *et al*., 2018; Li *et al*., 2019; Jiang *et al*., 2021). The CPV-2c variant was found to be the predominant antigenic variant in European countries, South America, and Australia (Decaro and Buonavoglia, 2012; Miranda and Thompson, 2016; Battilani *et al*., 2019; Hao *et al*., 2022). In India, CPV-2 was first reported in 1982 followed by 2a, 2b, new CPV-2a, new CPV-2b, and lately CPV-2c (Thomas *et al*., 2017; Kulkarni *et al*., 2019). As per the existing scenario, CPV-2a/new CPV-2a has established as a predominant strain of CPV-2 in the dog population of India (Kaur *et al*., 2016; Thomas *et al*., 2017; Kulkarni *et al*., 2019). However, there are only two reports of CPV-2c (Nandi *et al*., 2010; Gupta *et al*., 2018) from dogs in India with a very limited sample size.

Only one report is available on the molecular characterization of CPV-2 infection from a dog in Mizoram, India (Behera *et al*., 2020). However, sequence analysis of a large number of samples from all over the state of Mizoram is missing. Mizoram acts as a gateway to many exotic and endemic transboundary infectious diseases because of its porous international borders. This demands the use of PCR-based tools for the detection and differentiation of prevailing CPV-2 types for rapid diagnosis, timely intervention, and the formulation of control strategies. Further, as the CPV-2 evolution is ongoing, continuous surveillance is a pressing priority to prevent future outbreaks.

Keeping the above facts into consideration, the current study focused on molecular epidemiology, *VP2* sequence analysis, and phylogenomics of the CPV-2 virus which were detected in clinically affected dogs in Mizoram, India from 2014 to 2022.

Materials and Methods

Ethical approval

All animal experiments were carried out as per the guidelines issued by the Committee for Control and Supervision of Experiments on Animals (CPCSEA) and were approved by the Institutional Animal Ethics Committee with reference No. 1476/GO/Re/SL/11/ CPCSEA dated 12th June 2019.

Study area, population and sample collection

The state of Mizoram is one of the eight northeastern (NE) states of India. Within India's northeast region, it is the southernmost landlocked state, sharing borders with other NE states and sharing 722-km porous international borders with Myanmar and Bangladesh. It extends from 21°56'N to 24°31'N, and 92°16'E to 93°26'E. The state has an area of 8,139 square miles with moist tropical to moist sub-tropical climates (Jain *et al*., 2013). Between January 2014 and May 2022, a total of 202 samples (199 fecal samples, 2 vomit samples, and 1 tissue sample) were collected from dogs suffering from acute hemorrhagic gastroenteritis. These samples were obtained from district-level dispensaries, private clinics, and the Teaching Veterinary Clinical Complex of the College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Aizawl, Mizoram, India. Rectal swabs were collected and screened for CPV-2 infection by rapid antigen detection kit (Genbody, Korea) followed by confirmation through PCR (Supplementary Figure 1 (SF1)). Epidemiological information concerning age, sex, breed, season, vaccination status, and geographical location were collected from affected animals. Co-infections such as canine coronavirus, canine distemper virus, and canine adenovirus-1 were ruled out by a rapid antigen detection kit (Genbody, South Korea). Data concerning clinical signs of the affected dogs were scored as per Nguyen *et al*. (2006). Additionally, twelve age-matched healthy puppies, who were presented for routine health check-ups or vaccinations and found to be PCR-negative in their fecal samples, were included as controls for comparison. These comprised six mixed breed puppies (3 males and 3 females), two German Shepherds (1 male and 1 female), two Golden Retrievers (1 male and 1 female), and two Rottweilers (1 male and 1 female).

PCR screening and amplification of *VP2* gene fragment

Viral DNA was extracted from stool and vomit samples using the QIAamp Fast DNA Stool Mini Kit (Qiagen), and stored at -20°C until further use. All extracted DNA samples were screened for the presence

of CPV-2 using the Hfor (5´-CAG GTG ATG AAT TTG CTA CA-3´) and Hrev (5´-CAT TTG GAT AAA CTG GTG GT-3´) primer pair (Buonavoglia *et al*., 2001). Each PCR reaction was performed in a final volume of 25 µL containing 12.5 µL 2X PCR Master mix (Thermo Scientific DreamTaq Green PCR Master Mix (2X), USA, K1081, containing DreamTaq DNA Polymerase, 2X DreamTaq Green buffer, dNTPs, and 4 mM $MgCl_2$), 1 µL of each primer (10 µM), 2 µL of DNA template, and 8.5 µL of deionized sterile water. The thermo-cycling conditions were as follows: 5 min at 95°C (initial denaturation), 30 cycles of 30 s at 94°C (denaturation), 1 min at 50°C (annealing temperature), 1 min at 72°C (extension), followed by final extension (72°C for 10 min) and hold at 4°C. The DNA prepared from the CPV-2 vaccine strain (CanigenDHPPiL, Virbac India) was used as a positive control. The PCR amplification was carried out in an automated thermal cycler (Biorad T100, USA). The PCR-amplified products were resolved on 1.5% agarose gel in Tris Acetate EDTA (TAE) buffer and visualized under an ultraviolet transilluminator (Vilber Bioprint, France).

Cloning of PCR product

A total of 83 out of 103 positive PCR products were purified from the agarose gel using the GeneJet Gel Extraction Kit (Thermo Scientific, USA, Catalog number: K0691) followed by cloning. For gel extraction, the PCR reaction was carried out in a final volume of 50 µL, with each sample being processed in duplicate (total volume of 100 µL). The reaction mixture included 25 µL of 2X PCR Master Mix (Thermo Scientific DreamTaq Green PCR Master Mix), 2 µL of each primer (10 µM), 3 µL of DNA template, and 18 µL of deionized sterile water. The thermocycling conditions were the same as previously described.

All 83 gel-purified PCR products were cloned using the CloneJET PCR Cloning Kit (Thermo Scientific, K1232) following the manufacturer's instructions. This kit employs the pJET1.2/blunt cloning vector, which contains a lethal restriction enzyme gene disrupted by the ligation of a DNA insert into the cloning site. Consequently, only bacterial cells with recombinant plasmids can form colonies.

For cloning, the ligation mixture was prepared on ice to a final volume of 18 µL, consisting of 10 µL of 2X reaction buffer, 4 µL of purified PCR product, 3 µL of nuclease-free water, and 1 µL of DNA blunting enzyme. The mixture was briefly vortexed and incubated at 70°C for 5 min, followed by chilling on ice. Subsequently, 1 µL each of the pJET1.2/blunt cloning vector and T4 DNA ligase were added. The ligation mixtures were then directly used for transformation.

Fresh competent *E. coli* DH5α bacterial strains were prepared following Chang *et al*. (2017). The cells were transformed and cultured on Luria-Bertani Agar plates (Himedia India, Cat. No. M1151) containing ampicillin (100 mg/ml), and incubated overnight at 37°C to identify white colonies (Supplementary Figure 2 (SF2)). Ten white colonies (Supplementary Figure 3 (SF3)) per sample were selected for colony PCR (Supplementary Figure 4 (SF4)). Two representative PCR-positive colonies were then cultured in Luria-Bertani broth overnight at 37°C. 10 µL of LB broth cultured bacteria were stabbed into 1% Luria-Bertani Agar slants containing ampicillin (100 mg/ml) and incubated overnight at 37°C. The stab cultures showing bacterial growth (Supplementary Figure 5 (SF5)) were then sent to a sequencing facility for sequencing.

Genotyping of CPV-2 isolates

The recombinant plasmid carrying the correct insert, isolated from the representative clone was sequenced by Sanger DNA Sequencer. Nucleotide sequences thus obtained were edited and checked for vector contamination by using VecScreen tool of National Centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov/tools/vecscreen/). The sequences were then analyzed using BioEdit v 7.2.5 software (Isis Therapeutics, Carlsbad, CA, USA). The specificity of the sequences was then compared with those in the GenBank database using BLAST algorithm.

The nucleotide and amino acid sequences obtained for each sample were aligned with corresponding sequences available in GenBank using Clustal W of MEGA™ X software for Windows® (Tamura *et al*., 2011) with default parameters. The aligned sequences of partial *VP2* genes from all isolates were then submitted to Genbank for allotment of accession numbers.

Phylogenetic analysis

All the CPV-2 sequences obtained from this study along with the CPV-2 reference and vaccine sequences retrieved from GenBank were aligned using Clustal W of MEGA™ XI software using the maximum parsimony method and Tamura 3-parameter with Bootstrap consensus tree (1000 replications) (Saitou and Nei, 1987). A bootstrap value of ≥70% was considered significant for phylogenetic groupings (Xu *et al*., 2015). Deduced amino acid sequences of *VP2*, percentage homology, and differences were analyzed using MegAlign sequence alignment software (DNASTAR 6.0) (Tamura *et al*., 2011).

Selection pressure analysis of the samples in the study

The selective pressure on *VP2* genes of CPV-2 variants was analyzed by examining the ratio of non-synonymous (dN) to synonymous (dS) mutations (dN/dS) using Single-Likelihood Ancestor Counting (SLAC) via the Datamonkey web server (http://www.datamonkey.org, accessed on 1st July 2024). A dN-dS ratio of <1.0, 1.0, and >1.0 signifies negative, neutral, and positive selection, respectively. Additionally, further sequence analyses were conducted using Fixed Effects Likelihood (FEL), Fast Unconstrained Bayesian Approximation (FUBAR), and Mixed Effects Model of Evolution (MEME) on the same platform (Weaver *et al*., 2018).

Virus isolation

The main goals of virus isolation were to confirm viral identification (in addition to fecal PCR), assess pathogenicity (cytopathic effect), and maintain a virus bank for future studies, including vaccine development, diagnostic test development, and antiviral research. The Madin Darby Canine Kidney (MDCK) cell line, grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich®, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich®, USA), was used for virus isolation. Ten random CPV-2c PCR-positive fecal samples were filtered through a 0.45 µm syringe filter. When the monolayer in the 12-well cell culture plates was nearly complete, they were infected with 100 µL per well of the viral inoculum and incubated at 37°C for 1 h and 30 min to allow virus adsorption. After three washes, 1 ml of DMEM with 1% FBS was added, and the plates were incubated at 37°C for 3-4 days to observe

Table 1: Descriptive statistics of the dogs included in the study population (n=83)

Year	Dogs positive to CPV-2 DNA n=83 (%)	CPV-2a n=22 (%)	CPV-2b n=07 (%)	CPV-2c n=53 (%)	FPV* n=01 (%)	P-value
2014	08 (9.63)	06	02	00		0.001
2015	06 (7.22)	03	03	00		
2016	04 (4.81)	03	01	00		
2017	02 (2.4)	02	00	00		
2018	02 (2.4)	01	00	01		
2019	02 (2.4)	01	00	01		
2020	18 (21.68)	00	00	18	01	
2021	09 (10.84)	01	00	08		
2022	31 (33.34)	05	01	25		
P-value		0.213	0.658	0.001		
Sex						
Male	44 (53.01)	14	03	27	01	0.001
Female	39 (46.98)	08	04	26		
P-value		0.170	0.724	0.740		
Age						
1-3 month	30 (36.14)	06	02	22	01	0.001
4-6 months	42 (50.60)	13	05	24		
7-9 months	5 (6.02)	02	00	03		
>9 months	5 (6.02)	01	00	04		
P-value		0.001	0.247	0.001		
Breed						
Pure breeds	12 (14.45)	04	03	05		0.001
Mixed	67 (80.72)	16	03	48	01	
Mongrel	03 (3.61)	02	01	00		
P-value		0.001	0.555	0.001		
Season						
Winter (Nov. to Jan.)		09	03	02		0.001
Spring (Feb. to Apr.)		09	03	33		
Summer (May to Aug.)		04	01	07		
Autumn (Sept. to Oct.)		00	00	01		
P-value		0.288	0.555	0.001		
Origin						
Aizawl	48 (57.83)	08	02	38		0.001
Champhai	10 (12.04)	04	02	04		
Kolasib	05 (6.02)	01	00	03	01	
Lunglei	03 (3.61)	01	00	02		
Lawngtlai	06 (7.22)	02	00	04		
Serchip	04 (4.81)	01	03	00		
Chhimtuipui	03 (3.61)	02	00	01		
Mamit	03 (3.61)	02	00	01		
Saiha	01 (1.20)	00	00	01		
P-value		0.020	0.863	0.001		
Vaccination						
Unvaccinated	80 (96.38)	20	07	52	01	0.001
Incompletely vaccinated	03 (3.61)	02	00	01		
P-value		0.001	NA	0.001		

n: Total number of dogs included in the study group, NA: Not statistically analyzed, and * FPV was not included in the statistical analysis because of insufficient sample size

Table 2: Variant-wise clinical score (mean±SE) comparison against healthy control

Healthy control	CPV-2a	CPV-2b	CPV-2c	P-value
6.00 ± 0.01^{a}	$15.93 \pm 0.46^{b*}$	$15.57 \pm 0.64^{c*}$	$14.62 \pm 0.72^{d*}$	0.000

Superscripts $^{a, b, c, d}$ differed significantly (P=0.000) when clinical scores of CPV-2a, 2b, and 2c were compared against healthy control and superscript * differed non-significantly (P>0.05) when CPV-2a, 2b, and 2c were compared against each other

cytopathic effects (CPE).

The infected monolayers were harvested on day 3 post-inoculation (regardless of the presence of CPE) through three cycles of freezing and thawing, followed by centrifugation at 6000 ×g for 15 min in a refrigerated centrifuge. The supernatants were collected in microcentrifuge tubes and stored at -80°C until further use. Finally, the cell culture fluid from CPE-positive samples was subjected to PCR using the Hfor/Hrev primer pair for the confirmation of CPV-2 (Kaur *et al*., 2015).

Statistical analysis

The viruses detected were grouped based on the antigenic variant (CPV-2a, 2b, and 2c), and clinical data (Table 1) were analyzed using the Chi-squared ($\chi 2$) test when compared across the variants among the groups and one-way ANOVA was used to compare within the group. Variant-wise clinical score comparison (Table 2) was done using the Independent-Samples Kruskal-Wallis Test. The results were considered significant when P was ≤0.05. Statistical analyses were carried out using SPSS version 27.

Results

Molecular epidemiology data

Of the 202 dogs screened by PCR, 103 (50.99%) were found positive. Of the 103 positive samples, 83 samples were cloned and sequenced. Table 1 and Supplementary Table 1 (ST1) provide a summary of the descriptive statistics for the dogs in the study population, including isolate numbers, sample details, GenBank accession numbers, and antigenic variants. The median age of the dogs was found to be between 4-6 months (range 1.5 months to 5 years). The majority of the dogs (80/83; 96.38%) were found to be unvaccinated. Breed-wise prevalence was found to be highest among mixed-breed (80.72%) followed by pure-breed (15.66%) and non-descript dogs (3.61%). Season-wise prevalence was found to be highest during spring (67.46%) followed by winter (16.86%), summer (14.45%), and autumn (1.2%). Major clinical signs observed in dogs with CPV-2 (n=83) were anorexia (75%), moderate vomiting (65%), hemorrhagic watery diarrhea (64%), mild to moderate depression (61%), and mild dehydration (44%).

Sequencing, amino acid mutation, and variant-wise epidemiological data

To characterize the detected CPV-2 virus, the coding *VP2* gene sequence of 630 nucleotides was obtained from all the isolates. Based on the 426 amino acid (aa) residues of the deduced *VP2* protein, 53/83 (63.85%) CPV-2s were classified as 2c, 22/83 (26.50%) viruses were characterized as the 2a variant, 07/83 (8.43%) viruses belonged to 2b, and 1/83 (1.2%) was found to be FPV. The original CPV type 2 was not found. There was a statistically significant difference (P<0.01) across the year, gender, age, breed, origin, and vaccination status for CPV-2a, 2b, and 2c isolates when compared among each other. Similarly, within the group, 2a showed a statistically significant difference (P<0.05) concerning age, breed, origin, and vaccination status and 2c showed a statistically significant difference (P<0.01) concerning year, age, breed, origin, and vaccination status. No statistical association was found within the CPV-2b variant for the above-mentioned parameters (Table 1). Variant-wise clinical score comparison (Table 2) revealed a significant difference (P<0.01) against healthy control, however, a non-significant difference was observed when compared among the variants.

Several synonymous and non-synonymous mutations were noticed in all the types of CPV-2s which were unique to this study and the amino acid substitutions are summarized in Table 3. Non-synonymous mutation shown by 2c isolates were Gly05Ala, Phe267Tyr, Ser270Cys, Ala276Thr, Ser281Thr, Gln291Leu, Val294Leu, Asp298Glu, Val319Met, Glu363Gly, Ile324Tyr, Cys339Ser, Arg370Gln, Gly377Arg, Pro391Thr, Val415Ile, Leu462Pro, Asn464Tyr, and Lys466Asn. Similarly, non-synonymous mutation shown by 2a isolates were Phe267Tyr, Thr272Pro, Tyr290Phe, Arg291Lys, Lys331Met, Ile336Val, Gln387Lys, Met401Ile, His404Gln, Arg441Gly, and Val359Ala. Finally, non-synonymous mutations shown by 2b isolates were Tyr267Phe, Ala281Thr, Gly311Asp, Ile324Tyr, and Ala440Thr. The lone FPV isolate (MZ 17) did not show any non-synonymous mutation.

Phylogenetic analysis

The phylogenetic tree based on aa sequences is depicted in Fig. 1. The analysis revealed CPV-2, 2a, 2b, 2c, and FPV variants in distinct clades. Further, the original CPV-2, FPV, and vaccine sequences formed distant clades.

All the 2c isolates were grouped along with Chinese, Japanese, Italian, Taiwanese, and Thai 2c isolates from the database. Overall, they followed the same evolutionary pattern; however, there was a clear indication of separate lineages. The aa homology within the 2c isolates and with Chinese, Japanese, Italian, Taiwanese, and Thai 2c isolates ranged between 99.5%-100%.

In the present study, separate clusters of 2a under different clades were observed. MZ 134 clustered with Indian 2a isolate (KC713932). MZ 205 was found

Table 3: Amino acid variations in the CPV-2a, 2b, and 2c VP2 capsid protein of Mizoram isolates from the present study against reference and vaccine strains

	Amino acid position of *VP2* gene																																						
Isolate No.	05	267	270	272	276	281	290	291	294	298	311	319	324	331	336	339	354	359	363	370	377	387	391	401	404	415	426	440	441	462	464	466	Variant
MZ 01	a	F	-	-	-	-	-	-	-	-	-	-	I	-	-	-	R	-	-	-	-	-	-	-	-	-	-	A	-	-	-	K	2a
MZ 02	G	Y	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-	-	-	R	-	-	-	-	-	-	a	a	a	a	a	a	2c
MZ44	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2c
MZ60	a	-	-	-	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2a
MZ62	a	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2a
MZ64	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M	H	-	-	-	-	-	-	-	2a
MZ65	a	-	-	-	-	A	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2b
MZ70	a	F	-	-	-	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2a
MZ84	a	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	-	-	-	-	-	-	-	2a
MZ106	a	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2c
MZ134	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	2a
MZ137	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	-	-	-	N	A	-	-	-	-	2a
MZ142	a	Y	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	2b
MZ143	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2a
MZ187	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	-	-	-	N	A	-	-	-	-	2a
MZ188	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	-	-	-	N	A	-	-	-	-	2a
MZ54	a	Y	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	2b
MZ55	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	-	-	-	N	A	-	-	-	-	2a
MZ57	a	Y	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-	-	-		-	-	-	-	-	-	-	A	-	-	-	-	2b
MZ58	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	-	-	-	N	A	-	-	-	-	2a
MZ63	a	Y	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	2b
MZ65	a	Y	-	-	-	A	-	-	-	-	G	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	2b

MZ68	a	Y	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	2b
MZ78	a		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	-	-	-	N	A	-	-	-	2a
MZ89	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	2c
MZ93	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	2c
MZ98	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	2c
MZ56	a	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2a
MZ140	a	-	-	T	-	-	Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2a
MZ197	a	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2c
MZ203	a	-	S	-	-	-	-	Q	V	D	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	2c
MZ204	a	-	S	-	-	-	-	Q	V	D	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	2c
MZ208	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	2c
MZ213	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	-	-	2c
MZ216	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	2c
MZ217	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	2c
MZ218	a	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2c

a: Inadequate sequence data

Table 4: Amino acid divergence and homology percentage in the CPV-2a, 2b, and 2c *VP2* capsid protein of Mizoram isolates from the present study against vaccine strains

CPV-2 variant	Name of the vaccine	Divergence (%)	Homology (%)
CPV-2c	Megavac, India	3.4-5.7	94.3-96.7
CPV-2a		2.4-5.0	95.2-97.6
CPV-2b		3.4-6.0	94.3-96.7
CPV-2c	FJ222823 vaccine, 2b strain, Italy	1.5-2.9	97.1-98.6
CPV-2a		1.4-2.4	97.6-98.6
CPV-2b		1.4-2.4	97.6-98.6
CPV-2c	ON479057, NL-35-D vaccine, Italy	3.4-5.0	95.2-96.6
CPV-2a		2.9-3.7	96.3-97.1
CPV-2b		3.9-5.0	95.0-96.1
CPV-2c	ON479058, ON479059, ON479060, CPV-2 vaccine, Italy	4.0-5.0	95.0-96.0
CPV-2a		3.4-4.4	95.6-96.6
CPV-2b		4.4-5.0	95.0-95.6
CPV-2c	ON479061 CPV-2b BIO 12/B vaccine, Italy	1.9-2.6	97.4-98.1
CPV-2a		1.9-2.9	97.1-98.1
CPV-2b		1.9	98.1
CPV-2c	QQY98853 CPV-2a, VanguardPlus5-CV, Vietnam	3.4-5.0	95.0-96.6
CPV-2a		2.9-3.9	96.1-97.1
CPV-2b		2.4-5.0	95.0-97.6

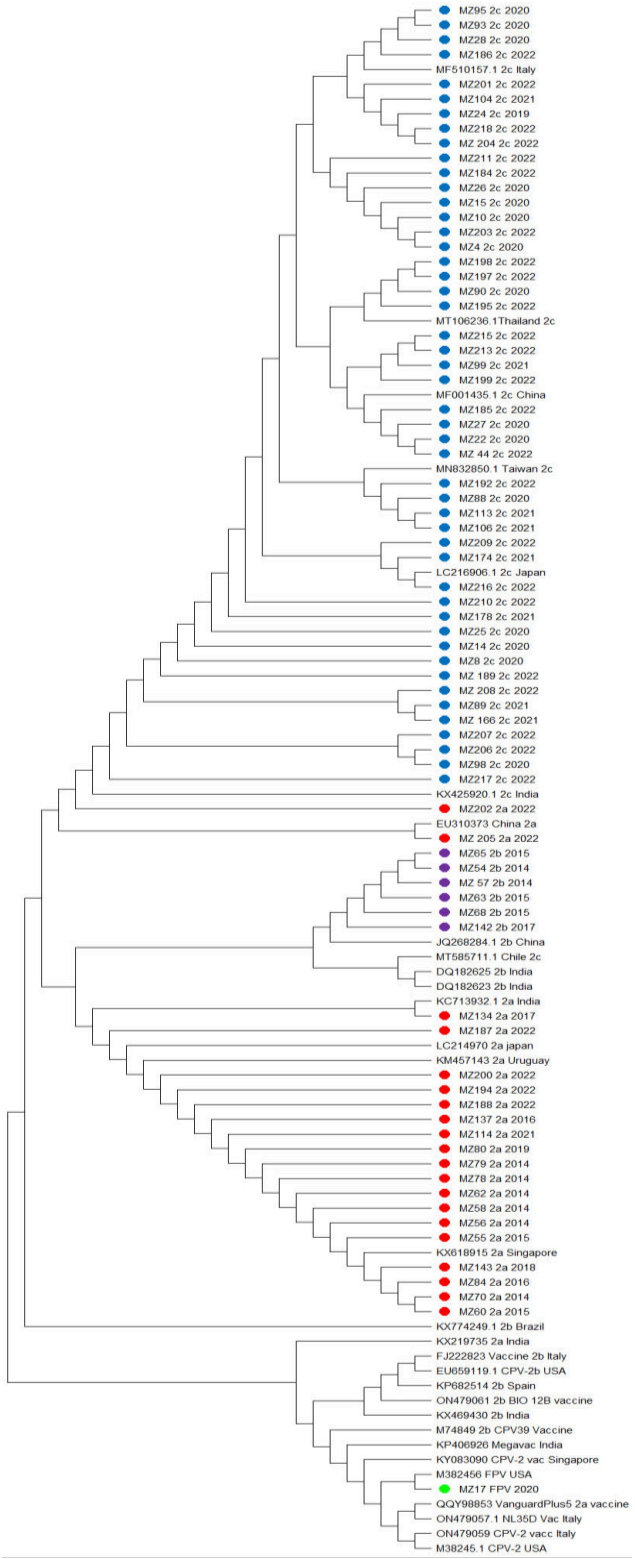

Fig. 1: Phylogenetic analysis of CPV-2 partial *VP2* sequences of Mizoram isolates using the maximum parsimony method with Bootstrap consensus tree (1000 replications). Drawn using MEGA version 11.0. The Tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Each isolate obtained in this study is indicated with initial MZ followed by sample number, year of isolation and strain identity. Blue, red, purple, and fluorescent green colours indicate CPV-2c, 2a, 2b, and FPV isolates obtained from this study, respectively

clustered with Chinese 2a isolate (EU310373). MZ 55, 56, 58, 62, 78, 79, 80, 114, 137, 188, 194, and 200 clustered with Uruguayan (KM457143) and Japanese (LC214970) 2a isolates. Another group of sequences (MZ 60, 70, 84, and 143) clustered with Singaporean (KX618915) 2a isolates. Overall, all the 2a isolates had aa homology ranged between 99.0% to 100% with each other and were ancestrally related to Indian, Singaporean, Japanese, Uruguayan, and Chinese 2a isolates (aa homology ranged between 98.9% to 100%).

2b isolates from the present study formed a single cluster with the Chinese 2b isolate (JQ268284) and had aa homology of 99% to 100% with each other.

The lone FPV isolates from the present study clustered with reference North American FPV (accession No. M38246) and had aa homology of 100%.

Further, all the CPV-2 variants from the current study showed aa homology of 95.0% to 97.1% with reference strains such as CPV-2 (M38245) and FPV (M382456). Amino acid divergence and homology percentage in the CPV-2a, 2b, and 2c *VP2* capsid protein of Mizoram isolates were analyzed against vaccine strains and are summarized in Table 4.

Selection pressures in the CPV-2 *VP2* protein

Selection pressure analyses carried out from the sequenced samples of the *VP2* gene yielded values of 0.260, 0.116, and 0.370 for CPV-2a, 2b, and 2c, respectively, indicating negative selection on the gene. However, specific positive selection sites were observed in CPV-2c at positions 24 and 67 (Table 5).

Virus isolation

In MDCK cell culture, cytopathogenic effects such as cell rounding and sloughing, characteristic of CPV-2 infection, were observed in 6 out of 10 CPV-2c positive samples at the third passage level (72 h of incubation) (Fig. 2). Furthermore, cell culture fluids from the infected cell line, 72 h post-infection, were processed to harvest viral DNA using the HiPurA® Multi-Sample DNA Purification Kit (Himedia India, Catalogue No. MB554-50PR) according to the manufacturer's instructions and the presence of CPV-2 was confirmed by PCR. However, CPV-2 cell culture confirmation by the immunoperoxidase method was beyond the scope of this study due to the unavailability of CPV-2 specific antibodies.

Discussion

The results of the present investigation suggested PCR positivity of 50.99% and reiterated its role as a quick and precise diagnostic technique (Kulkarni *et al*., 2019). The median age of the dogs was found to be between 4 and 6 months (range of 1.5 months to 5 years) and was consistent with an earlier report (Folitse *et al*., 2018). This might be attributed to factors such as increased intestinal epithelial turnover caused by changes in the bacterial microbiota, diet (weaning), and the interfering role of maternally derived antibodies (Castro

Table 5: Selection pressure analysis on the sequenced *VP2* gene of CPV-2

Variant	Sites of positive selection				Sites of purifying/negative selection			Mean dN/dS
	FEL[1]	SLAC[1]	FUBAR[2]	MEME[1]	FEL[1]	SLAC[1]	FUBAR[1]	
CPV-2a	None	None	183	None	Negative selection in six sites	None	Negative selection in six sites	0.260
CPV-2b	None	None	None	None	None	None	Negative selection in three sites	0.116
CPV-2c	None	None	None	24, 67	Negative selection in nineteen sites	Negative selection in one site	Negative selection in eleven sites	0.370

[1] P-value threshold of 0.1, and [2] Posterior probability of ≥0.90

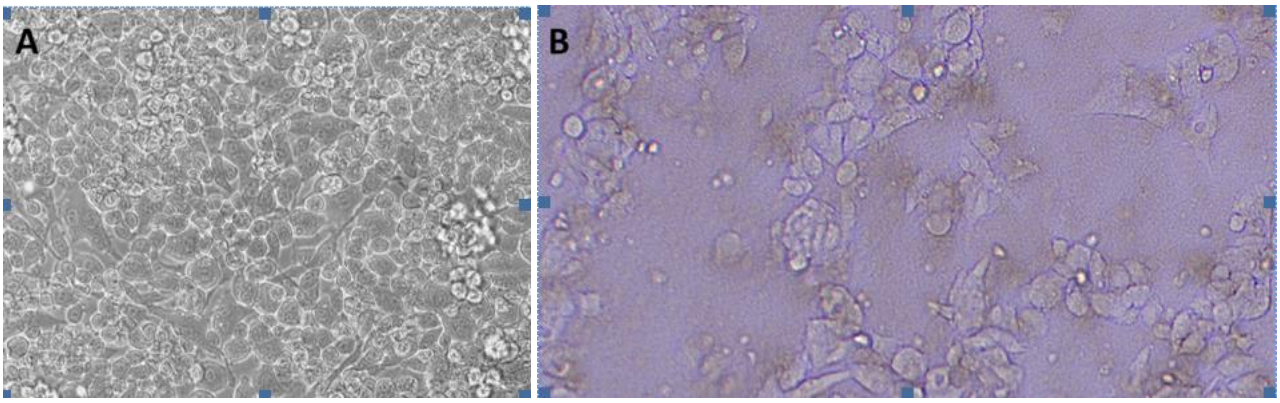

Fig. 2: Cytopathogenic effect of CPV-2c on MDCK cell culture. **A:** Uninfected cells**, B:** MDCK infected with CPV-2c (MZ 195) showing rounding and sloughing of cells (×200 magnification)

et al., 2013). The higher occurrence of CPV-2 in males (53.01%) compared to females (46.98%) in the present study was in agreement with earlier reports (Folitse *et al*., 2018; Chakraborty, 2022) which could be due to the over-presentation of male dogs during the study period (Castro *et al*., 2013).

The highest occurrence of CPV-2 in the mixed-breed dogs was in agreement with earlier reports which might be due to the over-presentation of that breed. In contrast, several authors found that pure-bred dogs had greater case fatality rates/risk factors than mixed-breed dogs, but failed to prove the same (Battilani *et al*., 2019). In conclusion, factors that make the pups prone to parvoviral infection irrespective of breed or sex are age, lack of protective immunity, overcrowding, intestinal parasites, and stressful environments (Schoeman *et al*., 2013).

The highest prevalence of the disease was seen during spring followed by winter and summer. Spring is characterized by significant diurnal temperature differences and variable weather and if the dogs' immune systems do not adjust to these temperature variations, this can reduce the dog's immunity causing an increasing prevalence of CPV-2 infection (Dong *et al*., 2020). CPV-2 infection occurs throughout the year and is more prevalent in the spring. This increased prevalence may be due to people spending more time walking their dogs outdoors during this season, thereby increasing the chances of dogs being exposed to viral pathogens in the environment (Zhao *et al*., 2016).

The most prominent clinical signs among CPV-2-affected puppies were anorexia, vomiting, and depression which were in agreement with earlier reports (Chakraborty, 2022). Numerous publications noted a striking variety in the clinical course of CPV-2 infection in dogs, ranging from subclinical infection to acute fatal illness. This variation was largely attributed to the age of infection, lack of protective immunity, stress level, and having a higher number of dividing cells (Schoeman *et al*., 2013).

In the current investigation, 96.38% of the dogs were found to be unvaccinated, which raised a major concern about the screening population's lack of disease-protective immunity. A statistical relationship was found between the vaccination status and the antigenic variants which might be attributed to lack of vaccination in the majority of the study population. However, confirming a specific antigenic variant might have evaded the vaccine-type immune response was beyond the scope of this study. To date, there has been no concrete evidence of a possible immune escape of CPV-2 variants (Battilani *et al*., 2019). Nevertheless, this study once again highlighted the importance of vaccination and awareness among the clients regarding compulsory vaccination in puppies.

CPV-2c was the predominant variant (63.85%) detected in the puppies in this study, which was in contrast to the other reports from different parts of India where either CPV-2a or new CPV-2a was found to be the predominant variant (Thomas *et al*., 2017; Kulkarni *et al*., 2019) along with occasional co-circulation of CPV-2b (Mukhopadhyay *et al*., 2014). Further, CPV-2a/2b is a prevalent strain circulating in Asia and CPV-2c has been occasionally detected in India as well as in this continent (Jiang *et al*., 2021). The first report of 2c from India was in the year 2006 followed by a hiatus of 12 years when the second report of 2c was found (Nandi *et al*., 2010; Gupta *et al*., 2018). It has also been reported in China

from 2009 to 2015 and in Taiwan between 2014 and 2016 (Chiang *et al*., 2016; Geng *et al*., 2017). From the present study, 2a was found to be the predominant variant up to 2017 till the first occurrence of CPV-2c in 2018 from the Aizawl district of Mizoram (MZ02, accession No. MT732073). From there onwards there was a shifting trend towards CPV-2c. The wide prevalence of CVP-2c from the present study was indicative of a shifting trend of CPV-2 antigenic variant in this part of the country. In comparison to 2a and 2b, this variant manifested the highest sequence variability which might be due to the virus's acquisition of multiple nucleotides and amino acid changes over time (Battilani *et al*., 2019). Considering the wide prevalence of this CPV-2c variant, a predominance of this variant is expected shortly.

Further, another unique finding of this study was the presence of FPV infection in a dog (OP778053) which had aa homology of 100% with North American FPV (accession No. M38246). There is sporadic information to date on FPV infection in dogs in the field. Recently, the transmission of FPV from cats to dogs was detected in Thailand, Italy, and Egypt (Charoenkul *et al*., 2019; Diakoudi *et al*., 2022) which was indicative of occasional FPV cases in dogs to date. This was because mutation of apical domain residues in host TfR was critical for controlling parvovirus binding (Charoenkul *et al*., 2019). So, veterinarians need to be vigilant as FPV-like viruses can infect dogs in the field, and monitoring of interspecies spread is required to understand the evolution dynamics, and potential host jumping of the virus.

All the CPV-2c isolates from the present study showed a synonymous mutation "Glu" to be present at residue 426 of the *VP2* protein (Decaro and Buonavoglia, 2012). Mutation such as Gly05Ala was also recorded in Chinese CPV-2c strains (Li *et al*., 2019). As residue 5 is one of the surface and core residues of the antigenic site of the virus, hence, the Gly05Ala mutation may alter its antigenicity and immunogenicity (Li *et al*., 2019). Phe267Tyr mutation was recorded both in CPV-2c and 2a isolates from this study which was earlier reported in Indian CPV-2a (Mukhopadhyay *et al*., 2014), Chinese CPV-2c and 2a (Li *et al*., 2019), Thai CPV-2a (Phromnoi *et al*., 2010) and Vietnamese CPV-2b strains (Nakamura *et al*., 2004). Tyr267Phe mutation was recorded in all the 2b isolates from this study and was also recorded from Indian (Gupta *et al*., 2018) and Chinese CPV-2b (Jiang *et al*., 2021) strains. This change may not affect the antigenicity of the virus as it is not exposed to the capsid surface (Jiang *et al*., 2021). The amino acid change Tyr324Ile was recorded in all the CPV-2b isolates in this study and was earlier reported from Indian CPV-2a isolates (Mukhopadhyay *et al*., 2014) and Chinese CPV strains (Jiang *et al*., 2021). The CPV host range is likely to be affected by mutation at residue 324 that affects the binding to the canine transferrin receptor (Cságola *et al*., 2014). One CPV-2c sequence from the current study had the mutation Arg370Gln, which was unique to this study and had not previously been reported from Indian 2c isolates. However, this mutation was reported from Italian, Chinese, Taiwanese, Thai, and Japanese 2c strains (Geng *et al*., 2017; Jiang *et al*., 2021). Residue 370 may be necessary for a conformational shift or may influence receptor binding via neighboring residues (Buonavoglia *et al*., 2001). Similarly, aa mutation 440 Thr→Ala was reported in all the CPV-2b strains from this study and had also been reported earlier in CPV-2a, 2b (Battilani *et al*., 2019) and 2c (Calderón *et al*., 2011) sequences from different parts of the world. The 440 residue sits at the peak of the threefold spike and is considered to be the primary antigenic site of the virus (Battilani *et al*., 2019). The remaining non-synonymous mutations were unique to this study and had not been reported to date. These non-synonymous mutations were recorded in the GH loop of *VP2* protein (267-498 residues located between βG and βH strands) of the virus which were exposed on the surface of capsid and likely to undergo mutation aiding in the evolvement of new variants (Kang *et al*., 2008). In addition, high variability of the surface antigenic sites may also be responsible for variation in host specificity (Kang *et al*., 2008). Although the functional impact of the majority of mutations is yet to be ascertained, nevertheless, the existence of all these mutations in the present study was ascribed to the high intrinsic rate of mutation coupled with persistent positive selection that would have contributed to the emergence of new CPV-2 variants.

Selective pressure analysis indicated that while CPV-2 strains were under purifying selection, positive selection sites were still found at two positions in the CPV-2c *VP2* gene. An increase in the dN/dS ratio has been observed over the years. The emergence of several positive selection sites in recent CPV-2c strains suggests mutations that are increasing in frequency across generations, enhancing the virus's fitness and aiding in evading the host immune response generated by traditional vaccines. This adaptability could be contributing to the virus's persistence in the current environment (Manh *et al*., 2021; Fu *et al*., 2022).

CPV-2 virus isolation is difficult and often produces a minimum visible cytopathic effect on cell cultures (Nakamura *et al*., 2004). The low frequency of adaptation of field isolates of CPV-2 in homologous cell cultures like MDCK has been previously reported. This could be because, in a natural infection, the virus typically targets the intestine, while MDCK cells are derived from renal tissue. Additionally, CPV-2 may require rapidly dividing cells in the mitotic phase to initiate infection, a condition that renal cells might not adequately provide. Other stimulating factors from the host that are present *in vivo* but absent under *in vitro* conditions might also play a role (Sharma *et al*., 2016). Nevertheless, the attempt to isolate CVP-2c in cellular cultures and characterization in this part of the country was the first of its kind which will help to achieve a better understanding of the current status of CPV-2 infection in the country.

The findings of the present study offered new insights into the evolutionary phylodynamics of CPV-2 in

Mizoram, India highlighting notable variations in the distribution of the CPV-2 variants. Further, it is important to note that CPV-2c variants have evolved to emerge as the predominant CPV-2 variant of dogs not only in this part of the country but also in other Asian countries in recent times. Looking into the current scenario of rapid spreading of this variant, the predominance of this variant is expected shortly. Although the circulating CPV-2 variants are generally under negative selection at *VP2* sites, the presence of two positive selection sites suggests selective pressure that may lead to the future emergence of new CPV-2c variants with varied antigenic and replication properties. It is also imperative to note the variation observed between field and vaccine strains which once again raises questions regarding the efficacy of vaccination and deserves continuous extensive surveillance. Further, the present finding will once again sensitize veterinary practitioners to pay more attention to both CPV and FPV infections in dogs.

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Conflict of interest

All the authors certify that there is no actual or potential conflict of interest about this article.

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