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Research Paper

Molecular epidemiology of *Canine parvovirus* shows CPV-2a genotype circulating in dogs from western India

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ABSTRACT

Canine parvovirus (CPV) has emerged as an acute pathogen of young canine causing haemorrhagic enteritis and myocarditis. It is widely distributed and underreported in India. Therefore the study was conducted to type the CPV circulating in western Maharashtra. The faecal samples ($n = 150$) from clinically ill dogs showing diarrhoea and vomition were collected and subjected to haemagglutination (HA) with porcine RBC's. The DNA was extracted from the samples showing HA titres above 64 and subjected for amplification of VP_2 gene fragment by PCR. The amplicons were subjected for restriction fragment length polymorphism (RFLP), sequencing and BEAST phylogenetic analysis. The results revealed 6% positivity by PCR. The RFLP results indicated single cleavage site for *ApaLI* and *Hinf*I with an exception of two sites for *Hinf*I. The nucleotide sequences showed nonfunctional nucleotide changes at different locations. The sequence analysis indicated that the nucleotide divergence within isolates under study was 0.00–0.42%, while the nucleotide homology was 99.58–100%. The most recent common ancestor was determined by molecular clock analysis using Bayesian methods. The sequence and phylogenetic analysis suggested the isolates as CPV-2a and KATN1 (KU866391, 2014) isolate from Tamilnadu, India as time to most recent common ancestor (TMRCA). The results revealed the circulating CPV in canines from western India as CPV2a genotype.

1. Introduction

Canine parvovirus (CPV) infection is an acute viral disease of dogs caused by *Canine parvovirus-2* (CPV-2) belonging to the genus Protoparvovirus, member of the Parvoviridae family, that has been included within the species *Carnivore Protoparvovirus 1*. The infection results into severe haemorrhagic enteritis and systemic disease, especially in pups with upto 100% morbidity and 10–91% mortality (Appel et al., 1978; Decaro and Buonavoglia, 2012; Mohyedini et al., 2013; Thomas et al., 2014). CPV-2 is named to differentiate it from CPV-1 which is also known as canine minute virus and was believed to be nonpathogenic until 1992. However, reports indicated less sever disease with lower mortality rates in dogs due to CPV-1 infection and considerable cases were reported from USA, Sweden, Italy, Germany and Japan (Pratelli et al., 1999; Mochizuki et al., 2002). Two clinical forms disease caused by CPV-2 are reported. The enteric form is characterised by vomition and diarrhoea and occurs in dogs of all age groups (Appel et al., 1978; Woods et al., 1980). Another form is characterised by myocarditis leading to heart failure. This form is reported in pups of age group below 3 months (Hayes et al., 1979). CPV-2 is one of the most pathogenic viruses of canines and was first recognised in 1977. Since then, it had spread throughout the world and established as enteric pathogen of domestic and wild canines. (Appel et al., 1979; Black et al., 1979). The virus is considered as host range variant of feline panleukopenia virus (FPV). CPV-2 has 3 antigenic variants: type 2a, 2b, and 2c, out of which 2a and 2b variants are distributed worldwide (Decaro et al., 2013). The disease has also been widely reported from India with high mortality rate (Sharma et al., 2012). The disease is reported in both, unvaccinated and vaccinated dogs. In India, sero-prevalence of CPV was first reported by Balu and Thangaraj (**1981**) in Madras and aetiological agent was confirmed as CPV-2 by Ramadass and Khader (**1982**). After that, a large number of CPV outbreaks have been reported from different parts of India (Savi et al., 2009; Nandi et al., 2010, 2010a; Mukhopadhyay et al., 2014). However, research on various aspects of dog health is neglected in developing countries like India, due to more emphasis on farm animals. To

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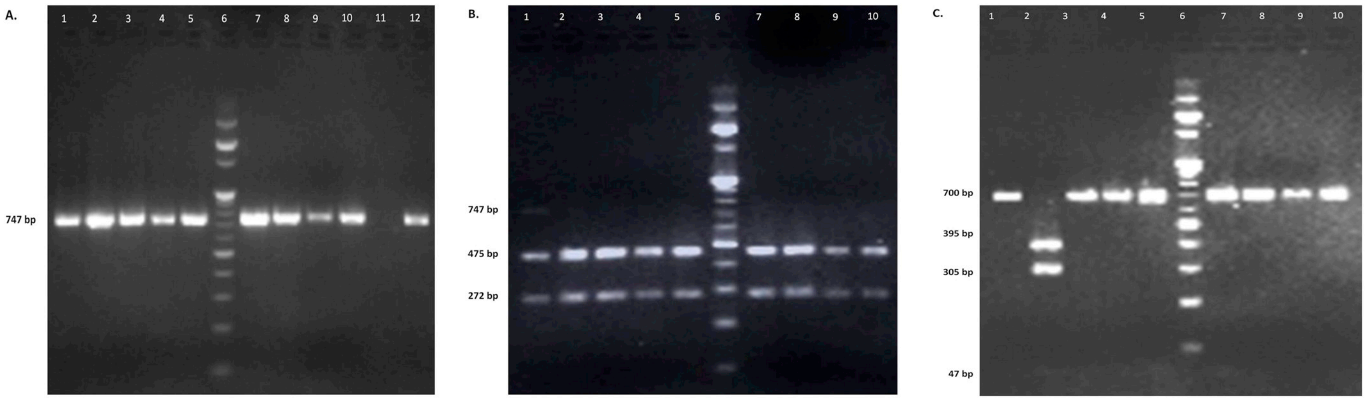

Fig. 1. A: VP$_2$ gene amplicons of CPV$_2$ isolates from western India, B: *ApaL*I digestion of VP$_2$ gene amplicons of CPV$_2$ isolates from western India, C: *Hinf*I digestion of VP$_2$ gene amplicons of CPV$_2$ isolates from western India.

address the canine health issues, research on contagious diseases causing heavy morbidity and mortality is a prime need. Considering heavy population of pet and stray dogs in the country and their indispensible role in human life as an embodiment of love and affection with diversified utility, it becomes necessary to address their health issues. In spite of heavy outbreaks and mortality (unpublished) by CPV, the causative agent was not sufficiently addressed to elucidate its molecular epidemiology from Western India. The available reports indicated presence of all the three variants i.e. CPV2a,b,c in India. However, these reports are based on limited samples obtained from Bareilly (Nandi et al., 2010) in north India and states of south India (Srinivas et al., 2013). Considering the knowledge gap about circulating CPV in western India and heavy morbidity and mortality in this region suspected for CPV infection, it becomes vital to utiliseand evaluate highly sensitive DNA based tools for detection and differentiation of locally circulating CPV types, which would eventually help in rapid disease diagnosis and to formulate control strategies, as the available vaccines may not be protective against CPV-2c strain. Similarly, if the new virus gains wider host range, deadly outbreaks could be observed like first emergence of CPV-2 in dogs.

In such circumstances it becomes necessary to undertake extensive studies epidemiological investigations of circulating strains of such potent pathogens. Therefore, the study was conducted to elucidate molecular insights and to study molecular epidemiology of locally circulating CPV from western India.

2. Material and methods

2.1. Sample collection

The faecal samples ($n = 150$) were collected from six different locations of western India during the year 2014–15. The dogs between 1 and 18 months of age showing clinical signs of illness like foul smelling watery diarrhoea, red colored faeces, vomition, anorexia, high temperature, depression, rough coat were considered for sample collection. The samples were collected in pre sterilized containers and transported on ice. The samples were preserved at -20 °C until further use.

2.2. Haemagglutination (HA) test

The faecal samples were weighed (1 g) and diluted in sterile PBS (pH 7.4) to obtain 10% (*W*/*V*) suspension. The resultant suspensions were clarified by centrifugation at 3000 rpm for 20 min at 4 °C. The supernatants were collected and used in HA test.

The HA test was performed as per O.I.E. protocol (O.I.E., 1992) with few modifications. The two fold dilutions from 1:2 to 1:4096 were obtained in 50 μl volume in chilled antigen diluent to which, equal volume of 1% porcine erythrocytes were added. The antigen and RBC controls were kept. The plate was incubated at 4 °C overnight and results were recorded.

2.3. Extraction of DNA and amplification of VP2 gene fragment

The total DNA was extracted from 200 μl diluted faecal samples ($n = 9$, showing high HA titres) by using QIAamp Mini DNA Kit (Qiagen, Germany) following manufacturer's instructions. The eluted DNA was used for polymerase chain reaction. The VP$_2$ gene fragment of viral DNA was amplified using specific primers, viz., 5′ tccagcagctatgagatc 3′ (F) and 5′ gatctgttggtagcaatac 3′ (R) targeting 747 bp region (Sakulwira et al., 2003). The PCR reaction was set up in 50 μl volume and consisted 100 ng of DNA template and 10 pmol of each primer with equal volume of 2× PCR Master Mix (Himedia Laboratories, India). The ingredients were mixed, brief spun and subjected for amplification in thermal cycler with 35 cycles of denaturation at 94 °C for 30 s., annealing at 58 °C for 15 s. and extension at 72 °C for 1 min. The DNA extracted from commercial vaccine was used as positive control and nuclease free water was used instead of DNA template in negative control. The amplicons were confirmed by their desired size in 1% agar gel electrophoresis and results were recorded in gel documentation system (Biorad, USA).

2.4. Restriction Fragment Length Polymorphism (RFLP)

Restriction digestion of the PCR products was performed as per the method of Sakulwira et al. (2001) by selecting restriction enzymes (*Hinf*I and *ApaL*I) from restriction map obtained from NEBcutter V 2.0 based on the sequences available in GenBank.

In brief, 1 μg of PCR amplicons were digested with 10 u of restriction enzyme at 37 °C for four hours followed by agar gel electrophoresis. The results were documented by using gel documentation system (Biorad, USA).

2.5. Sequence and phylogenetic analysis

The PCR amplicons were subjected for Sangers sequencing followed by sequence analysis. The obtained sequences and the database sequences retrieved from GenBank were analysed using BLASTn algorithm available at NCBI (http://blast.ncbi.nlm.nih.gov/Blast). The nucleotide sequences were aligned with 14 retrieved sequences of Indian, foreign and vaccine CPV using default parameters of muscle alignment implemented in MEGA 7.0 software (http://www.megasoftware.net/). Similarly, the deduced amino acid sequences of VP$_2$ were also aligned using muscle alignment with other related 14 amino acid sequences. Percentage homology and differences were analysed using Sequence Identity and Similarity Tool available at (http://imed.med.ucm.es/Tools/sias.html).

Table 1
Details of the CPV isolates and other global strains used for phylogenic analysis.

SN	Isolate ID	Genotype	Year	Origin	Accession no.
1	1/CPV/PBN	CPV-2a	2015	Western India	KX766013
2	2/CPV/PBN	CPV-2a	2015	Western India	KX766014
3	3/CPV/PBN	CPV-2a	2015	Western India	KX766015
4	4/CPV/PBN	CPV-2a	2015	Western India	KX766016
5	5/CPV/PBN	CPV-2a	2015	Western India	KX766017
6	6/CPV/PBN	CPV-2a	2015	Western India	KX766018
7	7/CPV/PBN	CPV-2a	2015	Western India	KX766019
8	8/CPV/PBN	CPV-2a	2015	Western India	KX766020
9	9/CPV/PBN	CPV-2a	2015	Western India	KX766021
10	KATN1	CPV-2a	2014	India	KU866391
11	SHMA2	CPV-2a	2014	India	KU866420
12	915H	CPV-2a	2013	India	KF366250
13	CHTN1	CPV-2a	2011	India	KU866402
14	Faizabad	CPV-2a	2009	India	KC713932
15	IIL/P15	CPV-2a	2005	India	DQ182620
16	IIL/P27	CPV-2a	2005	India	DQ182626
17	IIL/P1	CPV-2a	2005	India	DQ182612
18	IIL/P2	CPV-2a	2005	India	DQ182613
19	IVRI	CPV-2	2004	India	AJ698134
20	BJ104	CPV-2a	2014	China	KR869669
21	BJ480	CPV-2a	2014	China	KR869677
22	BJ219	CPV-2a	2014	China	KR869672
23	BJ131	CPV-2a	2014	China	KR869670
24	BJ137	CPV-2a	2014	China	KR869671
25	LSZA1	CPV-2a	2010	China	JQ996155
26	G5	CPV-2a	2009	China	KF482469
27	S6	CPV-2a	2009	China	KF482474
28	085/WH	CPV-2a	2008	China	FJ432717
29	S8	CPV-2a	2009	China	KF482475
30	1130	CPV-2b	2009	China	KF482478
31	GZ0201	CPV-2b	2002	China	GU569944
32	08/09	CPV-2c	2009	China	GU380305
33	QIACPV1404	CPV-2a	2014	Asia(Korea)	KP893078
34	H-Erd	CPV-2a	2012	Europe (Hungary)	KF539789
35	H-Halmai	CPV-2a	2012	Europe (Hungary)	KF539791
36	H-41	CPV-2a	2012	Europe (Hungary)	KF539802
37	H-27	CPV-2a	2012	Europe (Hungary)	KF539800
38	H36	CPV-2a	2012	Europe (Hungary)	KF539805
39	166	CPV-2b	2013	Europe (Spain)	KP682514
40	PT174/12	CPV-2b	2012	Europe(Portugal)	KR559892
41	434	CPV-2b	2007	Europe (Spain)	KP682525
42	140/05	CPV-2b	2005	Europe (Italy)	FJ005265
43	311/04	CPV-2b	2004	Europe (Italy)	FJ005262
44	217/07	CPV-2c	2007	Europe (Italy)	FJ005242
45	303	CPV-2c	2004	Europe (Italy)	FJ005209
46	388	CPV-2	2005	Europe (Italy)	FJ222824
47	M365	CPV-2a	2011	America (Uruguay)	KC196110
48	M315	CPV-2a	2011	America (Uruguay)	KC196113
49	M306	CPV-2a	2011	America (Uruguay)	KC196114
50	CO/898/10	CPV-2b	2010	America (USA)	JX475249
51	NM/1640/01	CPV-2b	1999	America (USA)	JX475241
52	E34/ECU	CPV-2c	2012	America (Uruguay)	KF149970
53	ME32/ECU	CPV-2c	2012	America (Uruguay)	KF149971
54	M169	CPV-2c	2011	America (Uruguay)	KC196104
55	GA/11	CPV-2c	2011	America (USA)	JX475238
56	VP2/Arg60	CPV-2c	2009	America (Argentina)	JF414823
57	M173	CPV-2c	2009	America (Uruguay)	KC196103
58	M152	CPV-2c	2008	America (Uruguay)	KC196105
59	M129	CPV-2c	2008	America (Uruguay)	KC196107
60	CO/28	CPV-2	2011	America (USA)	JX475231
61	ME/258	CPV-2	2011	America (USA)	JX475234
62	CO/1102	CPV-2	2011	America (USA)	JX475248
63	SC/182	CPV-2	2011	America (USA)	JX475233
64	U45	CPV-2	1997	America (USA)	AY742944
65	BR31–90	CPV-2	1990	America (Brazil)	DQ340414
66	6	CPV-2	1980	America (USA)	EU659117

The obtained sequences were subjected for recombination analysis using RDP4 tool (Martin et al., 2015) keeping default settings.

The sequences ($n = 66$) were further analysed for phylogenetic relationship using Bayesian time-scaled phylogenetic method (Nazia et al., 2016). Maximum clade credibility (MCC) phylogenetic tree was constructed by using Bayesian Markov Chain Monte Carlo (MCMC) analysis implemented in Bayesian evolutionary analysis sampling trees (BEAST) software package v2.4.2, and BEAST runs were performed by using CIPRES Science Gateway v.3.3 (https://www.phylo.org/portal2). Time to the most recent common ancestor (TMRCA) of CPV-2 strains was assessed using Bayesian inferences implemented in BEASTv2.4.2. Best fit nucleotide substitution model was determined by Akaike Information Criterion (AIC) using jModeltest2 software (Dorriba et al., 2012). An input ‘xml’ file for BEAST analysis was obtained by using Bayesian evolutionary analysis utility software Beauti v2.4.2 in which sequences were dated according to the year of isolation. Both strict and relaxed exponential molecular clocks (Drummond et al., 2006) were used in the analysis. Coalescent constant size and coalescent exponential tree prior were evaluated in the study. The best models were selected by means of Bayes factor (BF) test using marginal likelihood values obtained from Tracer 1.6.0 software. (http://beast.bio.ed.ac.uk/tracer). Three independent MCMC chains were run. During MCMC, the parameters were visited for 60,000,000 generations and sampled every 6000 cycles resulting in 10,000 trees (ESS > 200 for all the parameters estimated) and were assessed for their proper mixing, convergence and consistency by Tracer v.1.6.0 with 10% burn in. The 3 individual runs were combined using LogCombiner in the BEAST software package. The posterior tree distributions were summarised by using TreeAnnotator (http://beast.bio.ed.ac.uk/treeannotator) and exclusion of the first 10% of the trees as burn in. Node's having posterior probability higher than 0.5 were included. Phylogenetic tree with mean node heights were visualised in FigTree software v.1.4.2 available at (http://www.molecularevolution.org /software/phylogenetics/figtree). The uncertainty in the parameter estimates were assessed by 95% HPD (Highest posterior density- posterior distribution of phylogenetic trees is to rank the tree topologies by posterior probability and consider the smallest set of trees that represents at least 95% of the posterior probability) intervals. Bars depict the 95% HPD intervals of the time estimates. The scale on x- axis denotes years.

3. Results

3.1. Haemagglutination test

From a total of 150 faecal samples tested, 54 samples (36%) showed haemagglutination. The titre of 64 or less was recorded in 45 samples while, 6 samples showed titres range of 128 to 512 and 3 samples showed 1024 to 2048. The overnight incubation at 4 °C resulted into agglutination indicated by mat formation and negative samples yielded button formation. The positive control showed agglutination and negative control showed button formation.

3.2. Amplification of partial VP2 gene and restriction fragment length polymorphism (RFLP)

The total DNA extracted from faecal samples showing high haemagglutination titre were subjected for amplification of 747 bp fragment of VP_2 gene by PCR. The results revealed amplification of 747 bp fragments in all the 9 samples showing high HA titres, specific to the respective primer positions in the nucleotide sequence of VP_2 gene of CPV (Fig. 1 A). The results also revealed specific amplification in positive control. No amplification was recorded in negative control.

All the 747 bp PCR amplicons were subjected for restriction digestion using *ApaL*I and *Hinf*I restriction endonucleases. The RFLP results revealed single cleavage site for *ApaL*I (G,TGCA'C) in all the amplicons. The 747 bp VP_2 amplicons showed digestion into two fragments of size 475 bp and 272 bp with *ApaL* I enzyme. However, one amplicon (1/CPV/PBN) showed partial digestion. Digestion in this amplicon showed three fragments with two bands of size 475 bp and 272 bp and third band for undigested product of 747 bp size (Fig. 1 B).

The results also revealed single cleavage site for *Hinf*I (G,ANT'C) in all the amplicons except in 2/CPV/PBN (Fig. 1 C). The digestion yielded

Table 2
VP2 gene homology analysis for the nine Parbhani Canine parvovirus isolates and 14 global CPV isolates.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	1/CPV/PBN/02/2016		0.00	0.00	0.42	0.42	0.42	0.42	0.00	0.42	2.09	2.09	2.5	0.84	0.42	0.84	0.84	1.25	1.25	1.25	0.84	0.84	0.84	2.09
2	2/CPV/PBN/02/2016	100		0.00	0.42	0.42	0.42	0.42	0.00	0.42	2.09	2.09	2.5	0.84	0.42	0.84	0.84	1.25	1.25	1.25	0.84	0.84	0.84	2.09
3	3/CPV/PBN/03/2016	100	100		0.42	0.42	0.42	0.42	0.00	0.42	2.09	2.09	2.5	0.84	0.42	0.84	0.84	1.25	1.25	1.25	0.84	0.84	0.84	2.09
4	4/CPV/PBN/04/2016	99.58	99.58	99.58		0.42	0.42	0.42	0.42	0.00	2.5	2.5	2.92	0.84	0.42	1.25	1.25	1.67	1.67	1.67	1.25	1.25	1.25	2.5
5	5/CPV/PBN/05/2016	99.58	99.58	99.58	99.58		0.42	0.42	0.42	0.42	2.5	2.5	2.92	0.84	0.42	1.25	1.25	1.67	1.67	1.67	1.25	1.25	1.25	2.5
6	6/CPV/PBN/06/2016	99.58	99.58	99.58	99.58	99.58		0.42	0.42	0.42	2.5	2.5	2.92	0.42	0.00	1.25	1.25	1.67	1.67	1.67	1.25	1.25	1.25	2.5
7	7/CPV/PBN/07/2016	99.58	99.58	99.58	100	99.58	99.58		0.42	0.00	2.5	2.5	2.92	0.84	0.42	1.25	1.25	1.67	1.67	1.67	1.25	1.25	1.25	2.5
8	8/CPV/PBN/08/2016	100	100	100	99.58	99.58	99.58	99.58		0.42	2.09	2.09	2.5	0.84	0.42	0.84	0.84	1.25	1.25	1.25	0.84	0.84	0.84	2.09
9	9/CPV/PBN/09/2016	99.58	99.58	99.5	100	99.58	99.58	100	99.58		2.5	2.5	2.92	0.84	0.42	1.25	1.25	1.67	1.67	1.67	1.25	1.25	1.25	2.5
10	KM624023.1/FPV	97.91	97.91	97.91	97.50	97.50	97.50	97.50	97.91	97.50		0.84	1.67	2.09	2.5	2.92	2.09	2.5	2.5	2.5	2.09	2.09	2.09	0.84
11	EU018143.1|FPV	97.91	97.91	97.91	97.50	97.50	97.50	97.50	97.91	97.50	99.16		0.84	2.09	2.5	2.92	2.09	2.5	2.5	2.5	2.09	2.09	2.09	0.84
12	EF428258.1|MEV	97.50	97.50	97.50	97.08	97.08	97.08	97.08	97.50	97.08	98.33	99.16		2.5	2.92	3.34	2.5	2.92	2.92	2.92	2.5	2.5	2.5	1.67
13	DQ182612.1|CPV-2	99.16	99.16	99.16	99.16	99.16	99.58	99.16	99.16	99.16	97.91	97.91	97.50		0.42	1.67	0.84	1.25	1.25	1.25	0.84	0.84	0.84	2.09
14	KU866420.1|CPV-2a	99.58	99.58	99.58	99.58	99.58	100	99.58	99.58	99.58	97.50	97.50	97.08	99.58		1.25	2.5	1.67	1.67	1.67	1.25	1.25	1.25	2.5
15	FJ432717.1/CPV-2a	99.16	99.16	99.16	98.75	98.75	98.75	98.75	99.16	98.75	97.08	97.08	96.66	98.33	98.75		1.67	2.09	2.09	2.09	1.67	1.67	1.67	2.92
16	KP682514.1|CPV-2b	99.16	99.16	99.16	98.75	98.75	98.75	98.75	99.16	98.75	97.91	97.91	97.50	99.16	98.75	98.33		0.42	0.42	0.42	0	0	0	2.09
17	GU569944.1|CPV-2b	98.75	98.75	98.75	98.33	98.33	98.33	98.33	98.75	98.33	97.50	97.50	97.08	98.75	98.33	97.91	99.5		0.84	0.84	0.42	0.42	0.42	1.67
18	FJ005262.1|CPV-2b	98.75	98.75	98.75	98.33	98.33	98.33	98.33	98.75	98.33	97.50	97.50	97.08	98.75	98.33	97.91	99.5	99.16		0.00	0.42	0.42	0.42	2.5
19	FJ005262.1|CPV-2b	98.75	98.75	98.75	98.33	98.33	98.33	98.33	98.75	98.33	97.50	97.50	97.08	98.75	98.33	97.91	99.5	99.16	100		0.42	0.42	0.42	2.5
20	KC196104.1|CPV-2c	99.16	99.16	99.16	98.75	98.75	98.75	98.75	99.16	98.75	97.91	97.91	97.50	99.16	98.75	98.33	100	99.58	99.58	99.5		0	0	2.09
21	JX475238.1|CPV-2c	99.16	99.16	99.16	98.75	98.75	98.75	98.75	99.16	98.75	97.91	97.91	97.50	99.16	98.75	98.33	100	99.58	99.58	99.5	100		0	2.09
22	GU380305|CPV- 2c	99.16	99.16	99.16	98.75	98.75	98.75	98.75	99.16	98.75	97.91	97.91	97.50	99.16	98.75	98.33	100	99.58	99.58	99.5	100	100		2.09
23	M38245.1|CPV-2	97.91	97.91	97.91	97.50	97.50	97.50	97.50	97.91	97.50	99.16	99.16	98.33	97.91	97.50	97.08	97.9	98.33	97.50	97.5	97.9	97.9	97.9	

The lower left values are the percentage identities of the *VP2* gene among CPV isolates and the upper right diagonal of matrix shows divergence values; **1–9**: CPV isolates from the Parbhani (CPV/PBN/01 to CPV/PBN/09 respectively); **9–23**: CPV-2a, CPV-2b, CPV-2c, CPV-2, FPV and MEV isolates from India, China, USA and other countries retrieved from GenBank with accession numbers.

Table 3
Amino acid substitutions in CPV-2 variants.

Strain / Isolate	Amino acid substitutions			
	375	389	418	426
CPV-2	Asn	Thr	Ile	Asn
CPV-2a	Asp	Thr	Ile	Asn
CPV-2b	Asp	Thr	Ile	Asp
CPV-2c	Asp	Thr	Ile	Glu
1/CPV/PBN	Asp	Thr	Ile	Asn
2/CPV/PBN	Asp	Thr	Ile	Asn
3/CPV/PBN	Asp	Thr	Ile	Asn
4/CPV/PBN	Asp	Thr	Ile	Asn
5/CPV/PBN	Asp	Thr	Ile	Asn
6/CPV/PBN	Asp	Thr	Ile	Asn
7/CPV/PBN	Asp	Thr	Ile	Asn
8/CPV/PBN	Asp	Thr	Ile	Asn
9/CPV/PBN	Asp	Thr	Ile	Asn

two fragments of size 700 bp and 47 bp. The fragments of size 700 bp could be visible in the form of prominent band in 2% agarose gel. The other fragment was too small to be clearly visualised but showed migration below the gel loading dye. The amplicon from 2/CPV/PBN showed two cleavage sites yielding three fragments of size 395 bp, 305 bp and 47 bp.

3.3. *Sequence and phylogenetic analysis*

All the nine PCR amplicons were subjected for Sangers sequencing. The obtained nucleotide sequences were submitted to GenBank. The accession numbers are KX766013, KX766014, KX766015, KX766016, KX766017, KX766018, KX766019, KX766020 and KX766021. These sequences were analysed with other representative sequences of FPV, MEV, CPV-2, CPV-2a, CPV-2b, and CPV-2c retrieved from GenBank (Table 1). The divergence within CPV nucleotide sequences under investigation ranged between 0.00% - 0.42%, while the homology ranged between 99.58% - 100% (Table 2). The maximum divergence was recorded with MEV (2.5–2.92%) followed by FPV (2.09–2.5%), CPV2 (1.67–2.5%), CPV2b (0.84–1.67%), CPV2c (0.84–1.25%) and CPV2a (0.42–1.25%). Among CPV2a, the isolates showed 0–0.42% divergence with KU866420 (Tamilnadu) followed by 0.42–0.84% with DQ182612 (Telangana) and 0.84–1.25% with FJ432717 (China).

The sequence analysis indicated nucleotide changes in 2/CPV/PBN at position 3353 (G to T) and 3731 (A to G), in 3/CPV/PBN at position 3398 (A to T), in 5/CPV/PBN at 3353 (G to T), in 8/CPV/PBN at 3353 (G to T) and 3665 (G to A) and in 9/CPV/PBN at 3353 (G to A) and 3665 (G to A). The deduced amino acid sequences revealed presence of Asp at position 375 and Asn at position 426 in all the 9 sequences (Table 3).

The phylogenetic relationship of the isolates was analysed with 57 global CPV nucleotide sequences retrieved from GenBank. The time scaled Maximum Clade Credibility Tree computed (Nazia et al., 2016) is indicated in Fig. 2. The analysis grouped CPV 2, 2a, 2b and 2c variants in distinct clades. CPV-2c clade was placed closer to the CPV-2a clade. The original CPV-2 and FPV strains formed distant clades. No recombination event was recorded in the CPV-2a sequences of isolates from western India.

4. Discussion

Viral gastroenteritis is one of the major health issues of

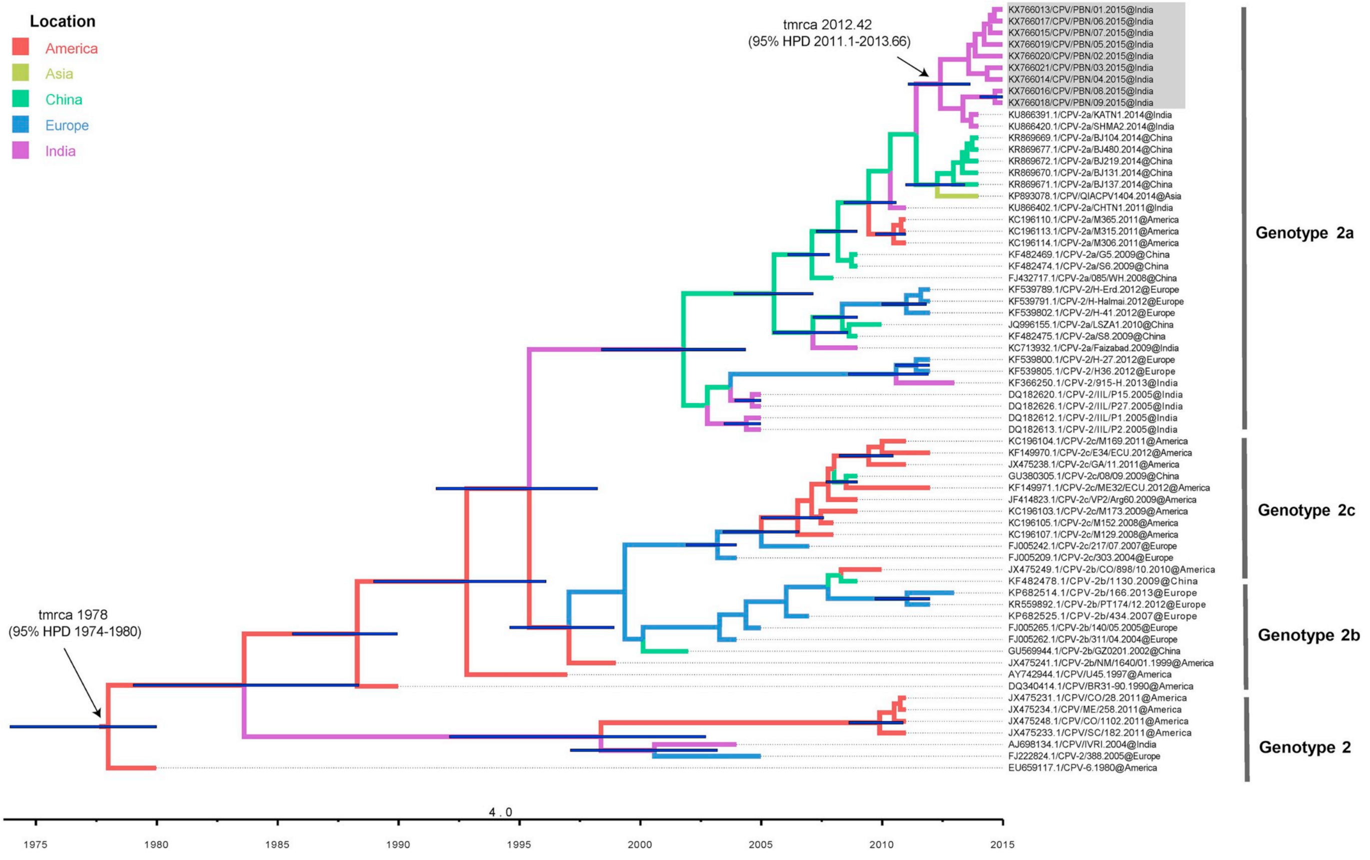

Fig. 2. Maximum clad credibility tree of CPV$_2$ isolates from western India. Tree derived with the best fit model (relaxed uncorrelated lognormal clock). Strains are colored with geographical annotations. Node bars in blue indicate 95% highest probable density. Strain sequences in the present study are highlighted in gray box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

unvaccinated and vaccinated canines and often results into heavy economic losses to pet owners. CPV is one of the major viruses causing gastroenteritis in puppies between 6 weeks and 6 months of age, however, the disease is nor restricted and can occur in any age group. The CPV infection is widely distributed in canines throughout the world and is well established in India. The present investigation elaborates the molecular insights of CPV from western India.

On HA test 36% samples revealed haemagglutination. The high positivity is indicative of wide spread of the disease. The high positivity (50.2%) was earlier reported by Banja et al. (2002). Though, 36% samples showed positivity by HA test, high titres of 64 and above are generally considered as positive for the CPV infection (Banja et al., 2002; Shashidhara and Kapil, 2009). Therefore, 9 samples showing HA titres of 64 and above were considered as positive while remaining 141 samples were considered as negative and thus, the true incidence was recorded as 6% in western India. Haemagglutination is one of the important biological properties exhibited by CPV. The results of the present study indicated binding of CPV to sialic acid N glycolylneuraminic acid receptors of nonhuman origin. This property of binding of virions to sialic acid receptors is essential factor for infection to the host cells. The haemagglutination property of the suspected faecal samples was indicative of presence of CPV. The samples showing titers above 64 were confirmed by PCR and RFLP. Thus, the HA test proved to be a specific, easy and inexpensive method for detecting CPV in faecal samples (Studdert et al., 1983; Kaur et al., 2015). Interestingly, two samples originated from vaccinated dogs also showed positive titres with disease specific clinical signs. These findings may be indicative of possible vaccine failure which may be attributed to worm load, poor nutritional status, non-maintenance of cold chain, faulty vaccination or interference of maternal immunity (Decaro and Buonavoglia, 2012; Chinchkar et al., 2014).

PCR could amplify VP_2 gene fragment of specific size as indicated by respective primer positions in the sequence. It has proved to be a rapid, sensitive and accurate diagnostic technique (Manoj Kumar et al., 2010). Further, PCR followed by sequence analysis can be used to differentiate CPV-2 antigenic variants (Nandi et al., 2010). The results of the present investigation suggested less positivity (36%) as compared to previous reports (Sakulwira et al., 2001; Parthiban et al., 2010; Mukhopadhyay et al., 2014; Nandi et al., 2015) who reported high positivity ranging from 58 to 78%. However, 10.4% positivity was reported by Mwalongo et al. (2014) in domestic dogs of Tanzania and Mittal et al. (2014) reported 6.7% positivity for CPV in Indian dogs. The PCR technique proved to be very sensitive as it detected CPV in all the nine samples having HA titers of 64 and above.

The RFLP results clearly indicated CPV specific digestion patterns. However, the pattern for 2/CPV/PBN was not in the accordance with other samples. This amplicon indicated an extra cleavage site for *Hinf*I, which could be due to nucleotide mutations in the sequence. RFLP of CPV VP_2 gene was stated to be used for differentiation of CPV variants (Sakulwira et al., 2001; Perez et al., 2012; Mukhopadhyay et al., 2014). However, the single nucleotide mutation, as detected in present investigation for *Hinf*I, can lead to changes in RFLP patterns, if they are located in cleavage sites of the restriction enzymes used for variant typing (Gupta and Singh, 2016), and may mislead the variant differentiation. Therefore, only PCR-RFLP may not be useful for characterization of CPV-2a. It should be complemented by sequence and phylogenic analysis.

The nucleotide sequence analysis indicated changes at different positions on VP_2 region. On analysis of deduced amino acid sequences, all these changes are found to be nonfunctional. Interestingly, a nucleotide change at position 3731 in 2/CPV/PBN resulted into an additional restriction site for *Hinf*I, which was not found in other sequences. However, though this mutation resulted into different RFLP pattern, it did not affected CPV variant. The CPV2, 2a, 2b and 2c variants could be differentiated based on two amino acids at position 375 and 426. CPV2 variants possess Asn instead of Asp at position 375 while CPV2a, 2b and 2c variants have Asn, Asp and Glu at position 426, respectively (Buonavoglia et al., 2001). All the 9 sequences under investigation showed presence of Asp at position 375 and Asn at position 426 indicating that they are CPV2a variants.

The phylogenic analysis indicated that CPV-2 evolved from FPV in the second half of 1970 decade which is also supporting previous report of Appel et al. (1978). The phylogenetic analysis placed CPV-2a sequences in three distant lineages. The clade of all nine isolates, under study, was clustered together with CPV-2a strains from Tamilnadu, India as lineage 1 (L1). Lineage 2 (L2) consisted CPV-2a from China and Asia, while, CPV-2a strains from America and China forms a somewhat distant lineage (L3). From the tree it is evident that CPV 2b and CPV-2c types formed two distinct clades. CPV-2c clade is closer to the CPV-2a clade. The original CPV-2 and FPV strains formed distant clades. The time scaled phylogeny was performed as per Nazia et al. (2016). HKY with gamma distribution was found best nucleotide substitution model as per jModeltest2. Uncorrelated relaxed lognormal clock wise Bayesian coalescent tree prior was choosen as the best fit model as they were favoured by Bayes factor. Model having low AICM (Akaike Information Criterion Model) values supposed to be better fit model. The AICM value of relaxed exponential clock with coalescent constant population model in the present study is 3451.30 ± 0.25. AICM values of remaining models studied are described in table 15. Difference in between these values is known as Byes factor. In this study relaxed exponential clock model with coalescent constant population model AICM value is low so it is better fit by Byes factor 23.48. KATN1 (KU866391, 2014) isolate from Tamilnadu, India was determined to be 4 years old (2012.42, 95% HPD, 2011.1–2013.66) and as time to most recent common ancestor (TMRCA) for all these nine isolates. The common TMRCA for all the strains under study was found to be present at 1978 (95%HPD, 1974–1980) which is in accordance with the report of Appel et al. (1978). The analysis indicated most recent evolution event in the mid of 2012 for CPV-2 isolates under investigation.

The results of the investigation confirmed that the CPV circulating in western India belongs to CPV-2a and are closely related to CPV-2a existing in other parts of India and they has evolved most recently in the mid of 2012. However, no recombination event was detected.

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Declaration of interest

None.

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