



Phylogenetic analysis of CPV isolate of Manipur*

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Canine parvovirus (CPV), a panzootic highly contagious and constantly evolving virus, is causing acute haemorrhagic enteritis and myocarditis in dogs. In India CPV isolates appear to evolve independently, and distinct geographical patterns of evolution could not be discerned (Chinchkar *et al.* 2006). The clinical diagnosis of CPV-2 infection is indecisive and methods based on detection of CPV-2 DNA by PCR are highly sensitive (Prashant Sharma *et al.* 2012). This study aims to delineate the molecular variant and phylogenetic analysis of CPV isolates from Manipur.

Faecal samples (34) were collected randomly from diarrheic and apparently healthy dogs during July 2005 to April 2007 from veterinary hospital and private clinics Manipur, along with the history for cross-sectional study via passive surveillance so as to determine the magnitude of canine parvovirus infection. Fecal specimens were homogenized (10% w/v) in phosphate buffered saline (PBS, pH 7.2) and HBSS (10% w/v) and clarified by centrifuging at 1,500g for 15 min and stored at -20°C until further use. Suspected samples were screened HA test as per Kumar *et al.* (2003). HA positive samples will serve positive control for differential PCR. DNA was extracted as per Sambrook *et al.* (2001). The type specific primer sequences of all PCR reaction selected from variable regions in the VP 1/VP2 capsid genes as reported by Pereira *et al.* (2000) were custom synthesized. The strategy used to type the samples was undertaken by an initial screening with the primers CPV2 and CPV-2ab. If the samples were positive with

primer CPV-2ab, it was amplified with CPV-2b to obtain type-specific results.

The PCR product of CPV-2b of 427 bp was first purified using purification kit and the cloned into pDrive cloning vector. Competent cells for transformation were prepared using DH5 α strain of *Escherichia coli* (Sambrook *et al.* 2001) and confirmed by colony PCR and restriction enzyme analysis using EcoRI enzyme. Sequencing was done at Delhi University of South Campus by automated DNA sequencer. The sequence was aligned against the other published CPV VP1/VP2 gene sequences by CLUSTAL METHOD using the Meg align programme of DNA Star software. The nucleotide sequence, phylogenetic maps and percentage homology were deduced and analyzed. Statistical analysis was done using Chi-square test (ChiSq.xlsx).

Faecal samples that agglutinate porcine erythrocytes with a hemagglutination titer of 1:16 and above were considered positive. This result was in agreement with the report of Kumar *et al.* (2003). Out of the total 34 faecal samples screened for CPV infection by PCR using 3 sets of primers, 18 samples were found positive, giving 52.9% positive percentage. Age-wise prevalence of CPV infection was significant ($P \leq 0.05$) with highest prevalence in the age group of 6–12 months (8 positive out of 10) followed by age group between 0–6 months (10 positive out of 22), dogs of age group of 12 months none of the 2 samples gave positive. From the present epidemiological results it can be deduced that the most susceptible age group was 6–12 months followed by 0–6 months and least above 12 months respectively. These findings can be supported by findings of Grigonis *et al.* (2002) which may be due to the protection seen just after birth to few months later due to the circulating maternal antibodies then susceptibility increases towards 6 months of age thereafter leading to a gradual decrease as the age advances. Significant difference ($P \leq 0.05$) was noticed in the prevalence of CPV season-wise with maximum prevalence during summer with 10 positive samples out of 16 followed by rainy season where 6 samples were positive out of 12 and least was found during winter with 2 positive samples out of 6 samples. Highest occurrence was noted during summer followed by rainy

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Sequence pair distances of Untitled, using Clustal method with Weighted residue weight table.
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Percent Similarity																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1		99.6	99.8	99.8	99.6	99.3	97.5	99.2	99.3	99.4	98.7	99.6	99.5	99.7	99.7	98.2	98.8
2	0.4		99.7	100.0	99.9	99.3	97.5	99.3	99.2	99.4	98.7	99.7	99.5	99.7	99.7	98.3	98.8
3	0.2	0.3		99.8	99.7	99.4	97.6	99.4	99.3	99.5	98.8	99.7	99.6	99.8	99.8	98.5	99.0
4	0.2	0.0	0.2		99.8	99.5	100.0	99.8	99.3	99.1	99.1	99.8	99.5	99.8	100.0	98.4	25.7
5	0.4	0.1	0.3	0.2		99.4	97.5	99.4	99.2	99.6	98.9	99.6	99.7	99.7	99.7	98.4	99.0
6	0.7	0.7	0.6	0.5	0.6		99.0	99.7	98.9	99.2	99.3	99.4	99.4	99.3	99.1	98.6	99.8
7	0.5	0.4	0.4	0.0	0.4	0.7		99.4	99.2	99.4	98.4	97.0	96.9	97.5	99.4	98.4	86.2
8	0.8	0.7	0.6	0.2	0.6	0.3	0.6		98.9	99.2	99.1	98.7	98.7	99.3	99.6	98.6	86.7
9	0.7	0.8	0.7	0.7	0.8	1.1	0.8	1.1		99.6	98.3	98.7	98.7	99.2	99.4	97.9	86.0
10	0.6	0.6	0.5	0.9	0.4	0.8	0.6	0.8	0.4		98.6	98.9	99.0	99.4	99.6	98.2	86.4
11	1.3	1.3	1.2	0.9	1.1	0.7	1.3	0.9	1.7	1.4		98.8	98.9	98.7	98.4	98.1	99.1
12	0.4	0.3	0.3	0.2	0.3	0.6	0.3	0.6	0.6	0.4	1.2		99.6	99.7	99.8	97.7	98.9
13	0.5	0.5	0.4	0.5	0.3	0.6	0.5	0.6	0.7	0.4	1.2	0.3		99.5	99.7	97.7	99.1
14	0.3	0.3	0.2	0.2	0.3	0.7	0.4	0.7	0.8	0.6	1.3	0.3	0.5		99.7	98.3	98.9
15	0.3	0.3	0.2	0.0	0.3	0.4	0.2	0.4	0.6	0.4	1.0	0.2	0.3	0.3		98.6	99.1
16	1.8	1.7	1.5	1.7	1.6	1.4	1.7	1.4	2.1	1.8	1.9	1.6	1.7	1.7	1.5		85.9
17	0.8	0.8	0.6	10.0	0.6	0.2	0.9	0.4	1.1	0.6	0.9	0.6	0.5	0.7	0.6	1.3	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17

Fig. 1. The percent homology and divergence of CPV isolate of Manipur (India).

season and winter, respectively, which also simulated with the findings of Grigonis *et al.* (2002), Rypula *et al.* (2004) and Houston *et al.* (1996), thus suggesting the reason to be more number of susceptible pups during summer, as majorities of litters being born during winter. Sex variation was not significant showing CPV prevalence in males and females showing 9 positive samples each out of 14 and 18 samples screened, respectively, giving statistically no significant results. The breed-wise prevalence of canine parvovirus was found highest in Doberman (4 out of 6) followed by Spitz (8 out of 11), GSD (4 out of 9), and it was least in mongrel (2 out of 8) which was observed to be significantly different ($P \leq 0.05$). The most susceptible breed is Doberman followed by Spitz, then GSD and least is mongrel, this result was in agreement with that of Houston *et al.* (1996).

The DNA templates when subjected to the primer sets CPV-2, there was no amplicon formation. The same samples when subjected to primer sets CPV-2ab yielded a product of 681bp product with distinct band in the gel electrophoresis. Subsequently those samples positive with CPV-2ab were finally subjected to PCR with the primer sets CPV-2b which yielded the specific amplicon of size 427 bp. Specific amplicon of size 427 bp and 681 bp was obtained using the primer sets for CPV-2b and CPV-2ab, respectively, and not with CPV-2 indicating that the strain identified is of CPV-2b. The PCR assay using differential primer showed that the variant prevalent at Manipur sample was CPV type 2b. The CPV-2ab primer set will amplify portion of VP1/VP2 gene of both CPV-2a and CPV-2b variants to yield a product size of 681bp. The CPV-2b primer set will amplify specific portion of VP1/VP2 gene unique to CPV. Differential amplification of CPV type 2b is ensured by single nucleotide variations in the very 3' end of each primer and these nucleotide variations (AG at 4062 and

GA at 4449) correspond to the strategic amino acid changes Asn-426 to Asp and Ile-555 to Val that determine the antigenic variation from type 2a to type 2b (Agbandje *et al.* 1995). The nucleotide sequencing of Manipur field sample confirms that it was CPV-2b variant. Similarly, Nandi *et al.* (2010) also reported CPV type 2b in field isolate to be more common in northern India, and vaccines strain to be CPV 2, while Chinchkar *et al.* (2006) reported CPV 2a to be more common in central and south India. Gene Bank accession no. EU118267 is assigned to the sequence fragment. When the nucleotide sequence was compared to various published sequences of CPV and its variants the Manipur field sample revealed to have 100 % homology and 0 % divergence with CPV-Japan 97-008, CPV Africa-3 and CPV-Polish isolate; 99.8 % homology and 0.2 %

Phylogenetic tree of Untitled, using Clustai method with seighted residue weight table.
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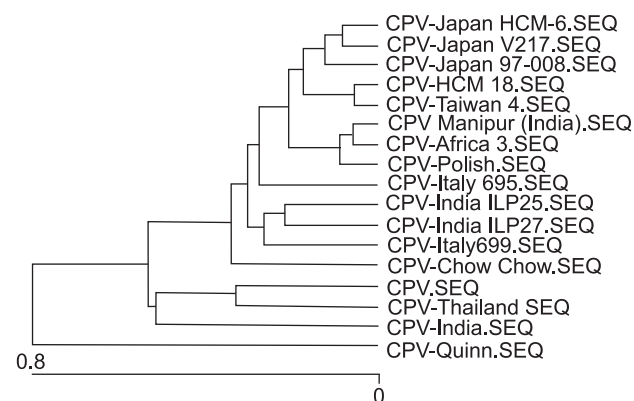


Fig. 2. The phylogenetic tree based CPV-2b isolate of field sample Manipur (India).

divergence with CPV-Japan V217, CPV-Italy 695, CPV-Chow Chow sequence, CPV-Taiwan4, CPV-Japan HCM-6; 99.5% homology and 0.5% divergence CPV-Italy695, CPV sequence; 99.3% homology and 0.7% divergence with that of CPV-India IILP25 isolate; 99.1% homology and 0.9% divergence CPV-India IIP27 isolate and CPV-India sequence; 98.4% homology and 0.6% divergence when compared with CPV-Quinn; and 25.7% homology with and 10% divergence CPV-Thailand. The phylogenetic tree (Fig 1) based on the nucleotide sequence revealed that the CPV-2b isolate of field sample Manipur comes in the monophyletic group form by CPV-Africa3, CPV-Polish, CPV-Taiwan4, CPV-Japan 97-008, CPV-Japan V217, and CPV-Japan HCM-6 (Fig. 2).

SUMMARY

This study was undertaken to determine the prevalent strain and epidemiological pattern of CPV isolates from Manipur. PCR was done with type-specific primers sets pCPV-2, pCPV-2ab and pCPV-2b. Out of 34 samples, 18 CPV isolates were obtained, all of which belong to CPV-2b. This is the first report of strain characterization of CPV isolates from Manipur (Eastern India) so it was further confirmed by sequencing of the cloned PCR product of 427 bp size. GeneBank accession no. EU118267 was assigned to the sequence. Comparative analysis of the sequence has shown to have cent percent homology with CPV-Japan 97-008, CPV Africa-3 and CPV-Polish isolates.

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