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CHARACTERIZATION OF CANINE PARVOVIRUS-2, CIRCULATING STRAINS IN HYDERABAD BY PCR AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM - PCR FROM CLINICAL CASES IN DOGS

PAWAR PURUSHOTHAM*, KALYANI P[1]. AND DHANALAKSHMI K[2]

[1]Department of Biotechnology, C.V.Sc, Hyderabad, India
[2]Department of Microbiology, C.V.Sc, Hyderabad, India

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Abstract– The canine parvoviral disease is one of the significant viral diseases of canines, mainly younger dogs responsible for vomition, acute hemorrhagic enteritis, myocarditis, and immunosuppression. Few years after its exposure CPV-2 was completely turned into two new antigenic variants designated CPV-2a and CPV-2b. A third variant, CPV-2c, became discovered in 2000 in Italy. This is a mutant of CPV-2b which involved the substitution Aspartate (Asp) 426 Glutamate (Glu) happening in a main neutralizing epitope of the capsid. So, the present study was attempted to study the virus's molecular characterization and phylogenetic analysis. Hundred faecal samples from the suspected clinical dogs exhibiting fever, and hemorrhagic enteritis referred to various private clinics in Hyderabad,were collected and subjected to isolation studies. In conventional PCR, sixty-seven samples yielded a single DNA amplicon of 681 bp with CPV-2ab primers. The same samples were further screened by CPV-2b specific primers and fifty samples yielded a single DNA amplicon of 427 bp to be confirmed as CPV-2b type. The final seventeen samples which were not responding to CPV-2b primers and responding to CPV-2ab primers were considered as CPV-2a type. All the 100 samples were further screened for CPV-2c type by CPV-555 primers that yield a single DNA amplicon of 583 bp which upon digestion with enzyme MboII generates two fragments of 500 bp and 83 bp for CPV-2cand remained undigested after MboII digestion, indicating that they are not of CPV-2c type but either CPV-2a or CPV-2b types as showed above. Phylogenetic analysis revealed that CPV-2a and CPV-2b of the current study had the closest sequence similarities with the Indian variants of CPV-2 available in GenBank.

INTRODUCTION

Canine parvovirus-2 (CPV-2) is a single-stranded DNA virus that is the most dreadful enteropathogen causing vomiting, acute hemorrhagic gastroenteritis, and myocarditis in dogs and young pups (Ahmed *et al*., 2018). The virus was designated as CPV-2, at its emengence in 70s, due to the existence of the minute virus of canines (MVC), known as CPV-1. During the 80s, the original virus (CPV-2) circulating worldwide acquired mutations, which led to the emergence of two antigenic subtypes (CPV-2a and CPV-2b) and the replacement of the prototype CPV-2 with the appearance of an additional antigenic subtype (CPV-2c) in 2000 in Italy (Clark *et al*., 2018). This is a mutant of CPV-2b which involved the substitution Aspartate (Asp) 426 Glutamate (Glu) occurring in a main neutralizing epitope of the capsid (Bounavoglia *et al*., 2001).

Some methods can be used to diagnose the disease, namely virus isolation in cell culture using Madin Darby Canine Kidney (MDCK), Crandell Rees Feline Kidney (CRFK) and A-72 cell lines, Haemagglutination (HA), Haemagglutination inhibition (HI), Agar Gel Precipitation Test (AGPT), Electron Microscopy, Indirect Fluorescent Antibody Test (IFT), Enzyme-Linked Immunosorbent Assay (ELISA) and Polymerase Chain Reaction (PCR) (Mochizuki *et al*., 1993). Because of this, the present research work has been taken up with the objectives, to identify Canine Parvovirus from clinical gastroenteritis cases using PCR, and Molecular characterization and phylogenetic analysis of the isolated strains.

([1]Assistant Professor and Head, [2]Professor)

MATERIALS AND METHODS

Screening of faecal samples by Haemagglutination test (HA)

A total of 100 faecal samples/rectal swabs (Table 1) were collected (from dogs exhibiting clinical signs of CPV viz., gastroenteritis, hemorrhagic enteritis, pyrexia, etc.) from Teaching Veterinary Hospital, College of Veterinary Science, Rajendranagar, Hyderabad and various Private clinics in Hyderabad. The faecal samples/faecal emulsified in 1mL of 0.1 M PBS of pH 7.4 and centrifuged at 6000 rpm for 15 min at 4vC. The supernatant was collected and stored at -20 °C until further use.90 µL of the processed faecal sample was treated with 10 µL of chloroform and mixed well. The mixture was kept at 4 °C for 10 min and centrifuged at 10000 rpm at 4 °C for 10 min.

Two-fold serial dilutions of 50 µL amounts of the chloroform-treated samples were made in 0.2 M Soreson's PBS of pH 7.0 in 96 well 'U' bottom microtitre plates. To each well 50 µL of 0.8 per cent, pig erythrocytes were added, mixed gently, and allowed to settle at 4°C for 4 hrs. One well, added with 50 µl of 0.2 M Sorenson's PBS of pH 7.0 and 50 µL of 0.8 per cent pig erythrocytes, served as cell control. The highest dilution of the sample showing complete haemagglutination was considered the haemagglutinationtiter.

Preparation of Sorenson's PBS (0.2M), pH 7.

Sodium chloride -8.8 g, Potassium phosphate (monobasic) - 14.97 g, Sodium phosphate (dibasic) 16.20 g, 950 ml distilled water was added and the pH was adjusted to 7.0 with 1N HCl or 1N NaOH., Bovine serum albumin - 1.0 g, Sodium azide (1:10000) - 0.1 g. The solution was made up to 1 litre with double distilled water, mixed slowly, and stored at 4ºC.

Preparation of Alsever's solution

Sodium chloride - 0.42 g, Glucose - 0.205 g, Citric acid - 0.005 g, Tri sodium citrate - 0.80 g, Double glass distilled water - 100 ml. The pH was adjusted to 7.2 and the solution was autoclaved at 121°C for 15 min and stored at 4°C until further use.

Preparation of Pig erythrocytes (0.8 per cent)

Packed pig erythrocytes of 0.8 ml were suspended in 100 ml of 0.2M Sorenson's phosphate buffered saline of pH 7.0, after three pieces of washing with the same solution.

Extraction of viral nucleic acid and Polymerase chain reaction (PCR)

The faecal samples/rectal swabs obtained from the suspected dogs were emulsied in 1 ml of 0.1 M PBS (pH 7.4) containing antibiotics (100 IU/ml Benzyl Penicillin, 100 µg/ml Streptomycin sulfate) centrifuged at 6000 rpm for 10 min at 4^0C. The supernatant was collected and filtered through a 0.22µ syringe filter and stored at -20 °C until further use.

100 µl of a faecal sample or freeze/thawed infected CRFK/MDCK cell culture harvest containing virus was used for template DNA preparation. The samples were boiled at 96 °C for 10 min and immediately chilled in crushed ice. The samples were then centrifuged at 12,000 rpm for 10 min at 4°C using a cooling centrifuge. The supernatant was then diluted 1:5 in nuclease-free water to reduce residual inhibitors of DNA polymerase activity and used as a template for PCR.

PCR was carried out using Emerald Amp® GT PCR Master Mix with a set of forward and reverse primers of CPV-2ab, CPV-2b, and CPV-555 primer which amplifies 681 bp, 427 bp, and 583 bp region in *VP2* gene segment respectively (Table 1). The primers were obtained in lyophilized form and were reconstituted with nuclease-free water to obtain 100 pmol/µl stocks. Stocks were made into 10 pmol/µl working solution and used for PCR.PCR was standardized for the primer set of CPV-2ab and CPV-2b as reported by (Nandi *et al*., 2010) and the primer set of CPV-555 as reported by (Bounavoglia *et al*., 2001).

Steps for PCR are as follows

1) The reagents were allowed to thaw completely. Then were mixed gently and spun briefly. The PCR master mix was prepared by scaling up based on the volumes listed below to the desired number of PCR reactions.
2) Using the template DNA, the PCR mixture was prepared as follows.

Reagent	Volume
Emerald Amp GT PCR Master Mix (2X Premix)	6.25 µl
Template DNA	5 µl
Forward Primer (10 µM)	0.5 µl
Reverse Primer (10 µM)	0.5 µl
dH_2O (Sterile distilled water)	0.25 µl
Total	12.5 µl

3) The above-mentioned contents were transferred to a 0.2 ml tube on ice. The PCR mixture was

mixed thoroughly by using the pipette and was spun briefly.

4) The tube was placed in a thermal cycler and the following cycling conditions for CPV-2ab and CPV-2b primer set were followed.

Initial denaturation	94 °C/5 min	
Denaturation	94 °C/30 sec	
Annealing	56 °C/1 min	35 cycles
Extension	72 °C/1min	
Final extension	72 °C/5 min	
Hold	4 °C/∞	

5) Cycling conditions for CPV-2C/555 primer set were followed.

Initial denaturation	94 °C/5 min	
Denaturation	94 °C/30 sec	
Annealing	52 °C/1 min	35 cycles
Extension	72 °C/1min	
Final extension	72 °C/5 min	
Hold	4 °C/∞	

6) Then gel electrophoresis was carried out at 80 volts for 1 hour and the PCR product was confirmed by comparison of migration distances with 100 bp DNA ladder, along with Positive Template Control and No Template Control (NTC); using gel documentation system.

PCR-RFLP

PCR products separated were purified from agarose gel by QIAquick® GelExtraction Kit as mentioned in kit protocol and PCR products were quantified by NanoDrop TM Lite Spectrophotometer.

Restriction digestion of the purified PCR products was carried out to confirm CPV-2c. The PCR products generated with primer pair CPV-555 (F)/CPV-555 (R) were then digested with enzyme MboII (New England Biolabs) that selectively recognizes the restriction site ''GAAGA'' (nucleotide 4062-4066 of the VP2 encoding gene) unique to CPV-2c only. After digestion at 37 °C for 12 h and enzyme inactivation at 65°C for 20 min, the digested products were analyzed in 2% agarose gel. The PCR products obtained from CPV-2c would be cut by MboII, generating two fragments of 500 and 83 bp, respectively.

8 µL of PCR products from each sample was digested with MboII enzyme in the reaction volume of 20 µL (Table 3.6).

Composition of RE digest

Reagent	Volume
MboII (5U/µl)	0.5 µl
Purified quantified PCR product	8 µl
1X NEB buffer	2 µl
dH_2O (Sterile distilled water)	9.5 µl
Total	20µl

The reaction mixture was spun for a few seconds for uniform mixing and then incubated in a water bath at 37 °C for 12 hours and then inactivated at 65 °C for 20 min.

SEQUENCING

PCR for sequencing

PCR was carried out using Jumpstart TMAccuTaq TM DNA Polymerase Mix with a set of forward and reverse primers of CPV-2ab, CPV-2b, and CPV-2c which amplifies 681 bp, 427 bp, and 583 bp region in *VP2* gene segment respectively.

Purification of PCR product for sequencing

To 1 volume of the PCR product, 5 volumes of buffer PB buffer (supplied with the kit) were added in micro-centrifuge tubes and mixed by inverting the tubes several times. The sample was then transferred to the QIAquick® spin column and centrifuged for 1 min at 13,200 rpm. Flow-through was discarded. 750 µl of Buffer PE was added to the spin column and centrifuged again for 1 min at 13,200 rpm. Flow-through was discarded and centrifuged for 1 min at 13,200 rpm. QIAquick® spin columns were placed in a fresh 1.5 ml micro-centrifuge tube. PCR product was eluted by adding 30 ml of nuclease-free water/EB buffer to the centre of the membrane, columns were kept stand still for 5 min and then centrifuged for 1 min at 13,200 rpm. Eluted product was quantified by NanoDrop TM Lite Spectrophotometer.

The DNA sequencing was done from Xcelris Labs Ltd, Gujarat using VP2 gene-specific primers as mentioned in Table 2 (S.No. 1 and S.No. 2) (for purified PCR products).

Phylogenetic analysis

The sequences of the VP2 gene of CPV-2 isolate obtained after sequencing were compared with other available sequences in GenBank using NCBI BLAST (www.ncbi.nlm.nih/gov/blast) and the phylogenetic tree was reconstructed with MEGA7

software to study the evolutionary relationship between CPV-2 serotypes.

RESULTS AND DISCUSSION

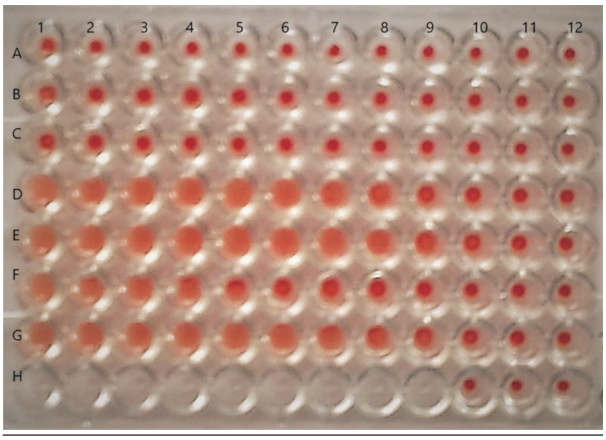
Preliminary screening of faecal samples for CPV-2 by HA Test

In the present study, isolation of the virus was attempted from faecal samples in 0.1M PBS of pH 7.4 as per the method of (Raj *et al*., 2010). Faecal samples were tentatively screened for CPV-2 by testing for haemagglutination with 0.8% swine RBC after chloroform treatment of faecal samples as per (Carmichael *et al*., 1980). Ten faecal samples were screened for CPV-2 (before attempting isolation) by haemagglutination with 0.8% swine RBC. 6 out of 10 samples caused haemagglutination with titers ranging from 32-1024 which were later confirmed by PCR (Fig. 4). These results indicating that haemagglutination of swine RBC by CPV-2 is a good test for preliminary diagnosis of CPV-2 and also for the selection of faecal samples, for further processing in cell culture, for isolation. Similar observations were previously made by (Carmichael *et al*., 1980).

Molecular Detection of CPV-2

100 faecal samples/rectal swabs obtained from suspected dogs were processed and DNA extraction was done. Commercially available vaccines for CPV-2 (Megavac and Canigen DHPPi) served as the positive control.

In conventional PCR using CPV-2ab primer pair (Nandi *et al*., 2010). 67 samples yielded a single DNA amplicon of 681 bp. All the samples found positive by CPV-2ab primers were further screened with CPV-2b primer pair (Nandi *et al*., 2010) and 50 samples out of 67 yielded a single DNA amplicon of 427 bp and confirmed as CPV-2b type. The remaining 17 samples which were not responding to the CPV-2b primer pair were considered as CPV-2a type. These results indicated a high prevalence of CPV-2b than CPV-2a, the same results are in agreement with Pereira *et al*. (2000), (Nandi *et al*., 2010), (Chinchkar*et al*.2006) and (Nandi *et al*., (2010) who observed dominance of CPV-2b over CPV-2a types.



S.No.	Sample ID (Table 3)	HA Titre
A	DNR-2	Negative (no matt formation)
B	TMR-4	32 (matt formation up to 5th well)
C	KDL-9	256 (matt formation up to 8th well)
D	PBP-4	1064 (matt formation up to 10th well)
E	TVH-4	1064 (matt formation up to 10th well)
F	TMR-33	1064 (matt formation up to 10th well)
G	KDL-13	1064 (matt formation up to 10th well)
H	Negative control	<2 (no matt formation)

Fig. 4. Results of HA test with faecal samples for CPV-2

PCR-RFLP

All the DNA samples (100 Nos.) were further screened for CPV-2c using CPV-555 primer pair (Bounavoglia *et al*., 2001) that would amplify all CPV-2 strains. The PCR products obtained with primer pair CPV-555 upon digestion with enzyme MboII would generate two fragments of 500 and 83

Table 1. Primers used for CPV-2 detection

S. No.	Forward and reverse primers	Primer Sequence (5′-3′) direction	CPV type amplified	Position of the genome	Annealing temperature and product size
1	CPV-2ab(F)	GAAGAGTGGTTGTAAATAATT	Both CPV-2a	3025-3045	56 °C
	CPV-2ab(R)	CCTATATAACCAAAGTTAGTAC	and CPV-2b	3685-3706	681 bp
2	CPV-2b(F)	CTTTAACCTTCCTGTAACAG	CPV-2b	4043-4062	56 °C
	CPV-2b(R)	CATAGTTAAATTGGTTATCTAC		4449-4470	427 bp
3	CPV-555 (F)	AGGAAGATATCCAGAAGGAGGT	CPV-2a,	4062-4066	52 °C
	CPV-555 (R)	GCTAGTTGATATGTAATAAACA	CPV-2b, CPV-2c		583 bp

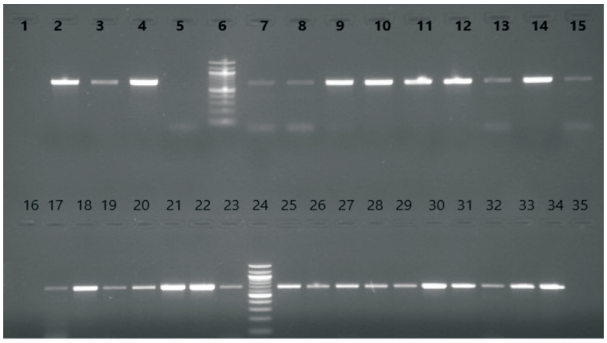


Fig. 7. Agarose gel electrophoresis pattern of CPV-2ab primers with an amplicon size of 681 bp.

Well (Fig 7)	Sample ID (Table 3)	Well (Fig 7)	Sample ID (Table 3)	Well (Fig 7)	Sample ID (Table 3)
1	Negative Control	13	DNR-17	25	TMR-10
2	TVH-4	14	DNR-18	26	TMR-11
3	DNR-6	15	DNR-19	27	TMR-12
4	DNR-7	16	Negative Control	28	TMR-14
5	DNR-8	17	DNR-20	29	TMR-16
6	100 bp ladder	18	DNR-21	30	TMR-18
7	DNR-9	19	TMR-1	31	TMR-19
8	DNR-10	20	TMR-3	32	TMR-20
9	DNR-13	21	TMR-4	33	TMR-21
10	DNR-14	22	TMR-5	34	TMR-23
11	DNR-15	23	TMR-9	35	TMR-24
12	DNR-16	24	100 bp ladder	-	-

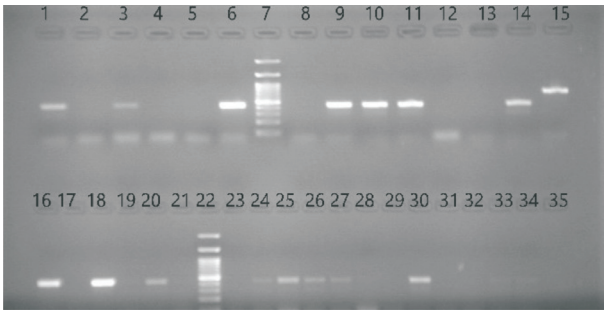


Fig. 8. Agarose gel electrophoresis pattern of CPV-2b primers with an amplicon size of 427 bp.

bp by selectively recognizing the restriction site GAAGA (nucleotide 4062 - 4066 of the VP2 encoding gene) unique to the CPV-2c type alone. PCR products separated were purified from agarose gel by QIAquick® Gel Extraction Kit. CPV types 2a and 2b remain undigested upon MboII digestion (Bounavoglia *et al*., 2001). Here, all products amplied by the CPV-555 primer pair remained undigested after MboII digestion indicating that they are not of CPV-2c type and may be either CPV-2a or CPV-2b types (already confirmed). Similar observations were made by (Parthiban *et al*.,

Well (Fig 8)	Sample ID (Table 3)	Well (Fig 8)	Sample ID (Table 3)	Well (Fig 8)	Sample ID (Table 3)
1	TVH-4	13	TMR-18	25	PBP-3
2	DNR-6	14	TMR-20	26	PBP-4
3	DNR-9	15	TMR-5 with CPV-2ab primers with an amplicon size of 681 bp.	27	PBP-5
4	DNR-10	16	TMR-21	28	PBP-13
5	DNR-11	17	TMR-22	29	PBP-14
6	TMR-9	18	TMR-23	30	KDL-3
7	100 bp ladder	19	TMR-24	31	KDL-4
8	Negative Control	20	TMR-25	32	KDL-8
9	TMR-10	21	TMR-28	33	KDL-9
10	TMR-11	22	100 bp ladder	34	KDL-13
11	TMR-12	23	Negative Control	35	KDL-15
12	TMR-17	24	PBP-2	-	-

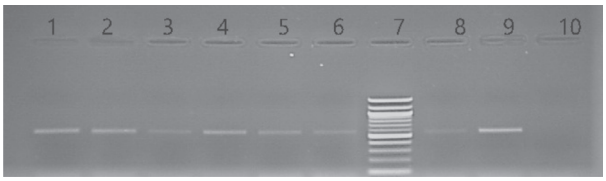


Fig. 9. Agarose gel electrophoresis pattern of CPV-555 primers with an amplicon size of 583 bp. None of the samples was digested by MboII.

Well (Fig 11)	Sample ID (Table 3)	Well (Fig 11)	Sample ID (Table 3)
1	TVH-4	6	TMR-19
2	DNR-7	7	100 bp ladder
3	DNR-9	8	PBP-4
4	TMR-5	9	KDL-13
5	TMR-10	10	NEGATIVE

2010)(Fig. 9).

VP2 Gene sequencing and Phylogenetic analysis of CPV-2

Phylogenetic analysis of nucleotide sequences of CPV-2a and CPV-2b obtained in this study aligned with 23 reference strains for CPV-2a and 32 reference strains for CPV-2b including vaccine strains like Megavac and CanigenDHPPi. CPV-2a/2b isolate was found to be phylogenetically closely related to new CPV-2a/2b strains of India and with reference CPV strains from various parts of the world using the Neighbour-joining method. Similar observations were made by (Srinivas *et al.,* 2013)**.**

One product of the CPV-2ab primer set with 681bp size and two products of CPV-2b primer set with 427 bp size were sequenced and generated forward and reverse sequence chromatograms were

initially edited with Chromas V 2.0 software and a consensus sequence was obtained. Using ClustalW, multiple sequence alignment was generated using MEGA 7.0 and sequence alignment were generated by comparing the nucleotide sequence of CPV-2 with other sequences of CPV downloaded from NCBI-Gen Bank. The evolutionary partial VP2 sequences of CPV-2 were inferred by using the Neighbour-joining phylogenetic tree method (Sheikh *et al*., 2017). Vaccines like Megavac and Canigen DHPPi were used as positive controls for phylogenetic analysis. According to the results of the present study, the Megavac vaccine strain appeared to be the best choice for the vaccination of dogs in India.

CPV-2a variant in the present study (PVNRTVU CPV-2a/2018) also showed 99 per cent nucleotide identity with the different variants in several geographical regions of the world, mostly with Indian isolates and with China, Japan, the Republic of Korea, Singapore, and Italy including vaccine strains. (Zhong *et al*., 2014) reported that in China, the predominant CPV-2 strains are CPV-2a and CPV-2b. Our results also showed that the predominant types of CPV-2 are CPV-2b (50) and CPV-2a (17) among 67 isolates in this study. According to our study, new CPV-2a or new CPV-2b has been the dominant CPV-2 in India for a long time, which is consistent with the results of other studies.

CPV-2b variants in the present study (PVNRTVU CPV-2b (1)/2018 and PVNRTVU CPV-2b (2)/2018) also showed 99 per cent nucleotide identity with Indian isolates and the phylogenetic tree detected five distinct clades (A-E). The isolates sequenced PVNRTVU CPV-2b(1)/2018 and CPV-2b(2)/2018 were under clade B and similar with India 1 IIL P25/ 2016 and India 6 P20/2016 and also with India 16 NDDB/2016, India 14 2016, India 15 2010, India 12 NDDB/2016, India 11 NDDB/2016, India 10 NDDB/ 2016, India 9 NDDB/2016, India 8 CPV-2b/2017 of clade A. vaccines strains vaccine-Megavac and vaccine-CanigenDHPPi were under clade D. When the samples were compared with the world and Indian isolates, it was observed that samples formed a separate node indicating regional genetic variation in the CPV-2. Similar results were reported by (Carmichael *et al*., 1980) who concluded that Indian isolates evolved independently from other isolates.

Unrooted Phylogenetic tree showing a relationship between PVNRTVU CPV-2b(1)/2018 and CPV-2b(2)/2018 isolate and other sequences of CPV-2b based on nucleotide sequences of VP2 region of CPV. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.02187253 is shown. The analysis involved 32 nucleotide Sequences.

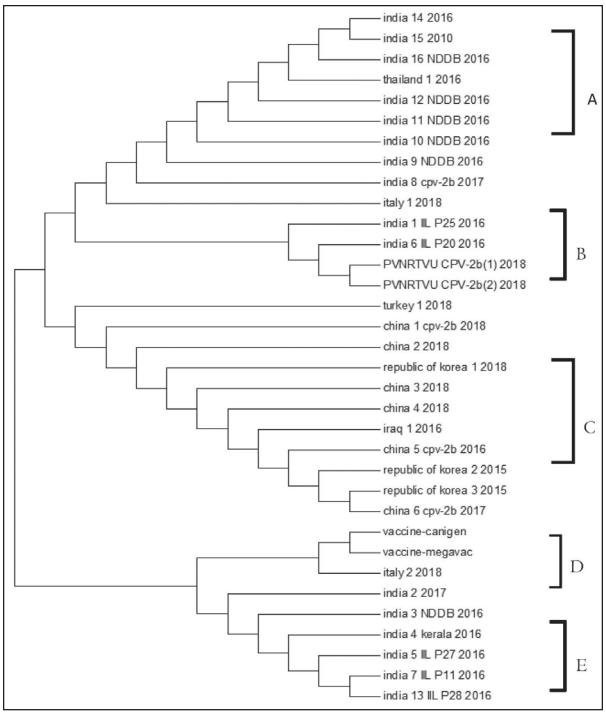


Fig. 10. Phylogenetic Analysis of Pvnrtvu CPV-2b(1)/2018 and Cpv-2b(2)/2018

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