A Comprehensive Molecular Survey of Viral Pathogens Associated with Canine Gastroenteritis

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Research Article

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Abstract

Viral pathogens account for the major aetiology of canine gastroenteritis. However, structured comprehensive studies on viral aetiology of canine gastroenteritis are sparse. To this end, 475 rectal swabs collected over a period of three years (2018–2021) from clinical canine gastroenteritis cases were screened for the presence of six majorly known enteric viruses (CPV-2, CDV, CAdV-2, CCoV, CaAstV, CRV), by real-time PCR. The overall incidence rate for each virus was found highest at 64.8% for CPV-2/2a/2b/2c (2a: 21.1%, 2b: 77.4%, 2c: 1.5%), followed by 8% for CDV, 7.2% for CaAstV, 5.9% for CCoV and 4.6% for CAdV-2 with 16.8% incidence for coinfections ranging from two to four viruses in different combinations; no incidence of CRV was reported. The whole-genome sequences of CDV, CCoV and CaAstV Indian isolates were reported for the first time and phylogenetic analysis was performed. This study highlights the need for relevant and routine vaccine prophylactic measures. It is alarming to note that 70.3% of animals vaccinated with DHPPIL were found positive for at least one virus. Hence, regular molecular analysis of the prevalent viruses is crucial to address vaccination failures.

Introduction

Dog has been the best common companion animal of all the domesticated animals and like humans, they are also prone to several diseases. Gastroenteritis, the irritation of the stomach and intestines is one among them resulting in vomiting and diarrhoea. This can progress quickly and can become very dangerous to the pet's health and, if left untreated, lead to death, especially in young animals. Gastroenteritis can occur for a variety of reasons *viz*., dietary indiscretion, tumours, metabolic disorders, toxins, and most important of all, infectious agents such as bacteria [1], parasites [2, 3], and viruses [4]. Viruses were reported to be detected in up to 60% of diarrhoeic faecal samples [5].

Canine parvovirus (CPV) [6], Canine distemper virus (CDV) [7], Canine adenovirus (CAdV) [8], Canine coronavirus (CCoV) [9], Canine astrovirus (CaAstV) [10], Canine rotavirus (CRV) [11] were frequently reported as a cause for viral gastroenteritis in dogs. CPV belonging to *Parvoviridae* family is highly contagious and was first identified in dogs suffering from severe hemorrhagic gastroenteritis and myocarditis as CPV-2 variant [12] [13]; later on replaced by three antigenic variants namely CPV-2a, CPV-2b, and CPV-2c [14–17]. CDV belonging to *Paramyxoviridae* family is responsible for high mortality rates in dogs worldwide [7]. Haemagglutinin gene of CDV was known to be evolved under genetic drift and was responsible for introduction of genetically different CDV strains [18]. It was reported that at least fifteen different lineages of CDV are circulating worldwide [19, 20]. CAdV belongs to *Adenoviridae* family and circulates as two distinct serotypes, CAdV-1 and CAdV-2 [8, 21]. Despite routine vaccinations, re-emergence of CAdV-2 was documented throughout the world [22]. CCoV belongs Alphacoronavirus 1 of C*oronaviridae* family, causes mild to moderate enteritis in dogs characterized by high morbidity and low mortality [9]. The Alphacoronavirus 1 also include transmissible gastroenteritis virus of swine, porcine epidemic diarrhea virus, feline coronaviruses, and human coronavirus 229E [23]. Canine astrovirus (CaAstV) belonging to Mamastrovirus of *Astroviridae* family [10] has been detected in multiple countries from diarrheic dogs [24–28]. It is mostly known to be associated with mixed viral enteric infections, especially in young dogs [28, 29]. CRV belongs to *Reoviridae* family and causes neonatal diarrhea [11].

The aim of the present study is to investigate and delineate the prevalence of major enteric viruses in stool samples from symptomatic dogs. Furthermore, first genome sequences of CDV, CCoV and CaAstV from Indian isolates are also reported along with their phylogenetic analysis.

Materials And Methods

Clinical sample collection and processing

Rectal swabs were collected from clinically ill dogs presented with gastroenteritis at Teaching veterinary clinical complex, College of Veterinary Science, Hyderabad and various private clinics in this region**.** The case histories of 475 samples screened in this study were presented in Table S1. Rectal swabs were collected over a period of three years (2018-2021). The swabs were homogenized in 3 ml of 0.1 M PBS (pH 7.4) containing antibiotics (100 IU/ml Benzyl Penicillin, 100 µg/ml Streptomycin sulphate), centrifuged at 6000 rpm for 10 min at 4^{o}C and the supernatant filtered through 0.22 µ syringe filter was used for further analysis.

Viral nucleic acid extraction and reverse transcription

The viral RNA was extracted using TRIZOL® reagent (Ref:15596018; Ambion®) and the cDNA was synthesized using PrimeScript first strand cDNA Synthesis kit (Cat No.6110A; TaKaRa) following the manufacturer's protocol. The viral DNA was extracted using phenol chloroform and isoamyl alcohol method as described by Sambrook and Russel [30].

Screening of clinical samples for viral etiology by real time PCR

The cDNA or viral DNA served as a template for PCR. The real time PCR was carried out with SYBR® Premix Ex TaqTM PCR Master Mix (Cat.No.RR420A; TaKaRa) in Step One Plus real-time PCR system (Applied Biosystems). The primers used for initial screening of six viruses in individual reactions were listed in the Table 1 [31–36]; along with the real-time PCR reaction conditions. The antigenic typing of CAdV was done into CAdV-1 and CAdV-2 based on the difference in melting temperature [32]. The antigenic typing for CPV-2/2a/2b/2c was done by a modified version of TARMS-PCR reported before [37, 38]

Isolation of virus in cell lines

DMEM containing 1% FBS was used for maintenance of Madin-Darby Canine Kidney (MDCK), A-72 (Canine fibroblast) or Epstein-Barr virus-transformed marmoset B lymphoblastoid (B95a) cell lines at 37^{o}C under 5% CO_2. The cells were kept in maintenance media during virus propagation. For virus isolation, the faecal samples were emulsified in PBS and the clear supernatant was used as a seed for virus culture. The virus isolation for CAdV-2, CPV-2/2a/2b/2c and CaAstV were carried out in MDCK cells, whereas for CDV and CCoV, B95a, and A-72 cells, respectively were used. The cells were incubated at 37^{o}C under 5% CO_2 and were observed for CPE such as granulation, rounding or detachment of cells in clusters disturbing the confluent monolayer. The cells were freeze-thawed three times and the viral supernatant was used as a seed for subsequent passages.

Whole-genome sequence analysis

The viral RNA was sent to the sequencing facility MedGenome Labs Ltd., Karnataka, India for whole genome sequencing. Briefly, the whole genome was sequenced using HiseqX (Illuminia). Around 12.3 Gb data was generated with 81 million reads for CaAstV and 14 Gb data was generated with 95 million reads for CDV and CCoV. The average Q30% of above 80% is considered. The reads were first aligned to canine genome (GCF_000002285.3_CanFam3.1) and the unaligned reads were then aligned to respective reference viral genome. *De novo* assembly was performed using metaspades to obtain scaffolds. The scaffolds were subjected to gene prediction using Prodigal and the predicted ORFs are subjected to Blastx. The sequences were deposited in the NCBI database using online BankIt submission form.

Phylogenetic analysis

Predicted viral genome was subjected to BLASTn. At least 15 sequences from the Blastn results were randomly collected based on percentage identity. Multiple sequence alignment was performed using MUSCLE algorithm in MEGAX software and was exported to MEGA file format. The phylogenetic tree was reconstructed using Neighbour Joining algorithm in MEGAX software by setting the test of phylogeny as bootstrap method and number of replicates as 1000 [39]. The clades were divided according to geographical distribution or as per relativeness in to different lineages/groups.

Results And Discussion

Most of the clinical presentations in dogs involve gastroenteritis, more often with viral aetiology. In the present study, we screened the rectal swabs from 475 clinically suspected dogs for the presence of either of six viruses namely, CPV-2/2a/2b/2c, CDV, CAdV-2, CCoV, CRV and CaAstV that are the most common aetiology of viral gastroenteritis.

Prevalence rate of gastroenteritis causing viruses

Being a highly sensitive, rapid, and specific technique that can detect low titre viruses, real-time PCR was successfully being used for molecular screening of clinical samples for viruses [31]. Initial screening for viral aetiology showed that around 71.6% of gastroenteritis cases were infected with either one or a mixture of the five tested viruses, whereas none of them tested positive for the presence of CRV (Table S1). Based on a previous molecular survey in symptomatic dogs, viral aetiology in gastroenteritis cases can go up to 93% [40]. Fig. 1a and Table S2 show the detailed prevalence rate for the viruses that tested positive upon initial

screening by real-time PCR. The overall incidence rate for each virus was found highest at 64.8% for CPV-2/2a/2b/2c, followed by 8% for CDV, 7.2% for CaAstV, 5.9% for CCoV and 4.6% for CAdV-2. Further, the antigenic typing of highly prevalent CPV-2/2a/2b/2c by TARMS-PCR revealed CPV-2b as the most prevalent antigenic type followed by CPV-2a, and CPV-2c (Fig. 1b and Table S3). It was previously reported that the prevalence rate for CPV-2/2a/2b/2c would be in the range of 50-70% [41, 42], for CDV to be around 2% [43, 44], for CaAstV in the range of 9-40% [28, 45, 46], for CCoV in the range of 8-65% [44, 47, 48], and around 60% for CAdV [4, 22]. The prevalence rate of viruses may depend on age of the dog (1m- 5years), geographical distribution and environmental factors. We observed that apart from CPV-2/2a/2b/2c all other infections occurred mostly in combination with other viruses, especially with CPV-2. The incidence rate for coinfections ranging from two to four viruses in different combinations accounted for 16.8%. It was previously reported that the mixed infections with different viral combinations was much common in dogs with gastroenteritis [3, 40, 49–51]. In conjunction with previous reports [3, 40, 49], we suggest that the gastroenteritis cases may be screened for mixed infections for effective treatment. Most of the pet owners failed to provide proper history as there was no pet health record maintained with them. Of the animals for which the vaccination history was available, it was interesting to note 70.3% of the animals vaccinated with DHPPIL were found positive for at least one of the virus in question. This observation alerts the need to focus on updating the vaccine with circulating strains of the viruses. Moreover, it highlights the need for regular molecular and serological screening of prevalent viruses to address the vaccination failures.

Recovery of virus isolates in cell lines

Because of the presence of mixed infections, especially with CPV-2, a highly virulent virus [52], it was difficult to obtain virus isolates from MDCK cells alone. Hence for isolation of CDV and CCoV we used specialized cell lines such as B95a [53] and A-72 [54]. The virus isolates were recovered by infecting the respective cell lines as described in previous section. We observed CPE after 5 dpi for CDV in B95a cells, 3 dpi for CPV-2/2a/2b/2c, CaAstV and 5 dpi for CAdV-2 in MDCK cells, 5 dpi for CCoV in A-72 cells (Fig. 2).

Whole genome sequencing based evolutionary dynamics

Worldwide, there were limited reports of whole-genome sequences of CDV, CCoV and CaAstV isolated from dogs [55–57]; and we found no reports from India, till date. Hence, the whole genome of the three viruses (CDV, CaAstV, CCoV) was sequenced, extracted and the ORF regions were predicted. The total genomic length was found to be around 15.6 kb for CDV, 6.5 kb for CaAstV, 29 kb for CCoV which was nearly similar to that of the previous reports [58–60]. We found six ORFs (N, M, F, H, L, P) for CDV; three ORFs for CaAstV (ORF-1a, 1b, 2); and two overlapping ORFs coding ORF-1a and RdRp genes along with nine other genes (M, S, E, N, ORF-3a, 3b, 3c and ORF-7a, 7b) for CCoV as similar to earlier reports [29, 60, 61]. The complete sequences along with protein-coding regions were deposited in the GenBank database of NCBI using BankIt submission and the accession numbers are MT905031 for CDV, MT894143 for CaAstV and MT955604 for CCoV. The whole-genome sequence of CDV was clustered with Asia-1 lineage that was distant from vaccine lineage (Fig. 3a), CaAstV was clustered with group III that consists of China and Brazil isolates when divided into three groups based on the relatedness (Fig. 3b). CCoV was clustered with group II that consists of China and Taipei isolates when divided into two groups based on the relatedness (Fig. 3c). A previous study suggested that all wild type strains of CDV clustered in a similar way to the groups obtained as in H gene analysis, which is normally used to identify geographically distinct CDV lineages [57]. We observed a similar grouping in this study. Further, we also noticed that all the wild type strains are genetically distant to vaccine strains [62, 63]. For CaAstV and CCoV, we could only find very limited reports of whole genome based phylogenetic analysis based on geographical distribution. A study reported a similar observation for CaAstV where one of the strains they sequenced got clustered with China isolates [55]. Another study reported that the CCoV isolates from five provinces of China during 2018-19 clustered with the earlier isolates from China similar to our observations [56].

Conclusions

From the present study we conclude that all the three antigenic variants of CPV-2 (CPV-2a/2b/2c), CDV, CaAstV, CAdV-2 and CCoV were involved in canine gastroenteritis during the period 2018-21. CPV-2b was found to be the most prevalent antigenic variant of all the gastroenteritis viruses surveyed in this study. We notice that majority of the gastroenteritis cases presented to the clinics were infected with at least one virus and the number of viruses infecting each dog range from one to four. To our knowledge, this is the first study to report the whole genome sequences of the CDV, CAstV and CCoV viruses isolated in dogs from India. The phylogenetic analysis shows the isolates were distant to that of the vaccine strains. We strongly emphasize on the need for developing a relevant vaccine with circulating variants of viruses for canine gastroenteritis.

Declarations

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Competing Interest

The authors have no relevant financial or non-financial interests to disclose. The authors confirm no conflict of interest arising from the work. All the authors accepted the order of authorship.

Author contributions

AD, MRT performed the experiments, involved in whole genome sequence data analysis and submission. BB, HK, AR performed the sample collection and molecular screening. VKG involved in whole genome sequence data analysis and submission, drafting the manuscript. YNR was involved in conceptualization of study and mentored the work. KP was involved in conceptualization of study, mentored the work, and analyzed the data and drafting of manuscript. All the authors proofread the manuscript and accepted the final version of the draft.

Data availability

My manuscript has data included as electronic supplementary material.

Ethics Approval

This is an observational study. The Institutional Animal Ethics Committee has confirmed that no ethical approval is required.

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Tables

Table 1 Primers used for screening of viruses by real time PCR

S. No.	Primer name	Primer sequence	Target gene	Virus	Amplicon size	PCR conditions	Reference
1.	CPV-F	5'AAACAGGAATTAACTATACTAATATATTTA3'	VP-2	CPV	90	Initial denaturation at 95°C for 5 min followed by 4 cycles of 95°C for 30 sec and 60°C for 1 min. Melt curve was set starting at 50°C to 95°C with a ramp speed of 1%.	[31]
	CPV-R	5'AAATTTGACCATTTGGAT AAACT3'					
2.	CAdV-F	5'AGTAATGGAAACCTAGGGG3'	E3	CAdV	166		[32]
	CAdV-R	5'TCTGTGTTTCTGTCTTGC3'					
3.	CDV-F	5'AGCTAGTTTCATCTTAACTATCAAATT3'	N	CDV	87		[33]
	CDV-R	5'TTAACTCTCCAGAAAACTCATGC3'					
4.	CaAstV-F	5'GTACTATACCRTCTGATTTAATT3'	ORF1b	CaAstV	293		[34]
	CaAstV-R	5'AGACCAARGTGTCATAGTTCAG3'					
5.	CCoV-F	5'TTGATCGTTTTTATAACGGTTCTACAA3'	M	CCoV	99		[35]
	CCoV-R	5'AATGGGCCATAATAGCCACATAAT3'					
6.	CRV-F	5'TTAGATACTACAAGTAATGGAATCGGATGT3'	VP7	CRV	76		[36]
	CRV-R	5'TGGGTGTCATTTGATACAACTTCA3'					

Figures

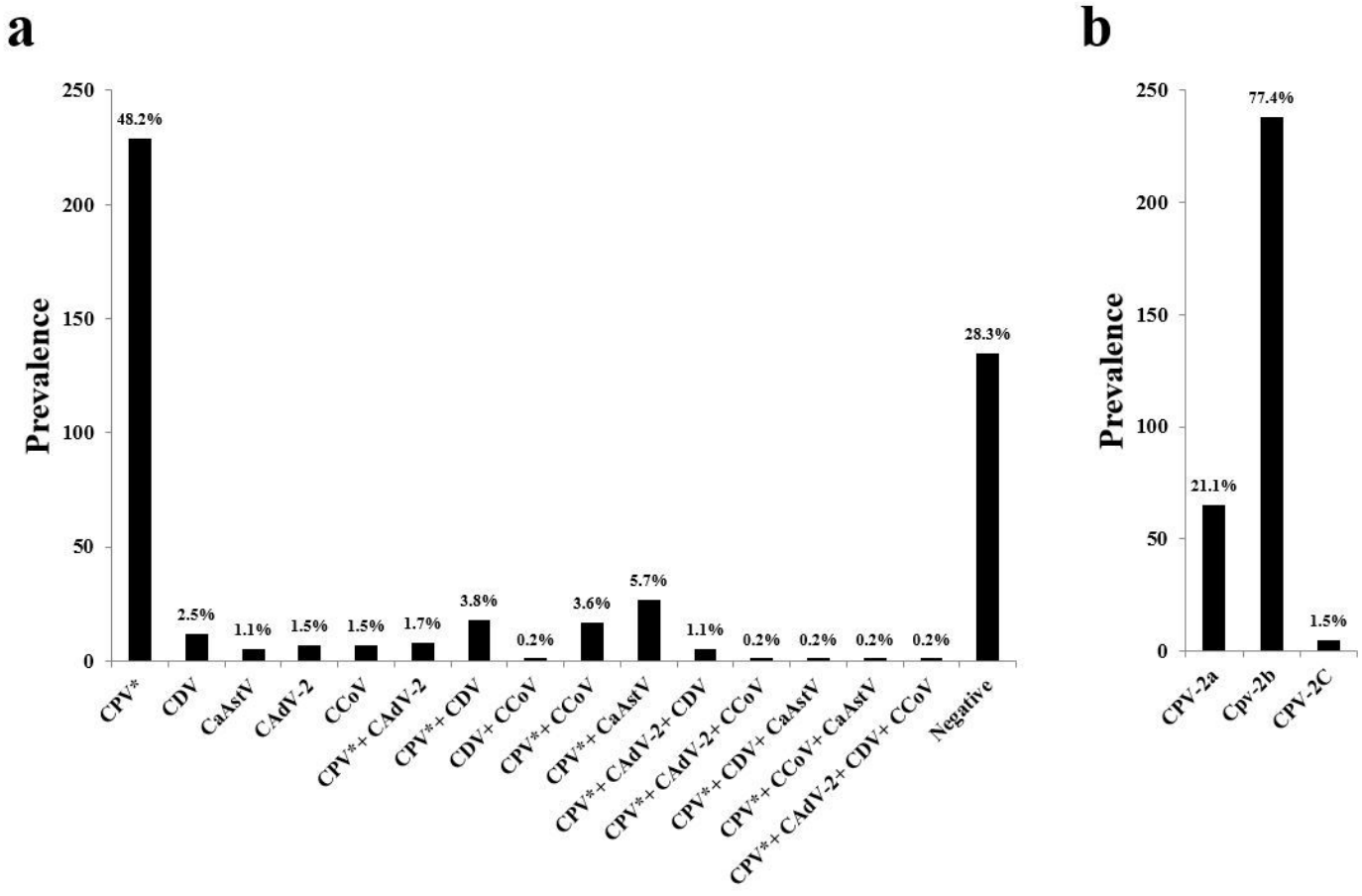

Figure 1

Prevalence of canine enteric viruses. a) The prevalence of different canine enteric viruses during the year 2018-21 in the Hyderabad region of Telangana state in India was depicted with bar diagram. The length of the bar indicates number of samples tested positive (Y-axis) for each virus either individually or in mixed infection (X-axis). The percentage above the bar indicates the prevalence rate for that particular virus. CPV* indicates either CPV-2/2a/2b/2c. b) The antigenic prevalence of CPV-2/2a/2b/2c during the year 2018-21 in Hyderabad region of Telangana state in India was depicted with bar diagram. The length of the bar indicates number of samples tested positive (Y-axis) for respective antigenic variant (X-axis). The percentage above the bar indicates the prevalence rate for that particular antigenic variant.

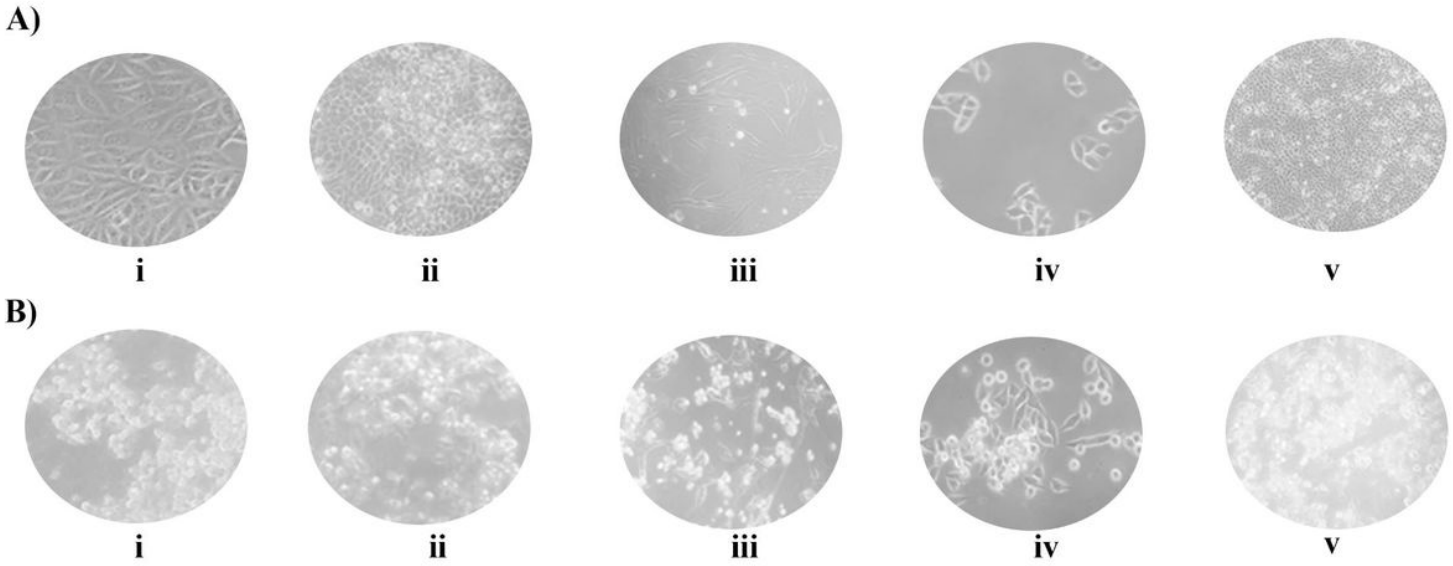

Figure 2

Isolation of canine enteric viruses. Panel A shows healthy cell culture images taken at 20X (total of 200X) magnification whereas panel B shows virus infected cell culture images taken at 20X (total of 200X) magnification. From left to right (i) MDCK/CPV (ii) MDCK/CAdV-2 (iii) A-72/CCoV (iv) B95a/CDV (v) MDCK/CaAstV.

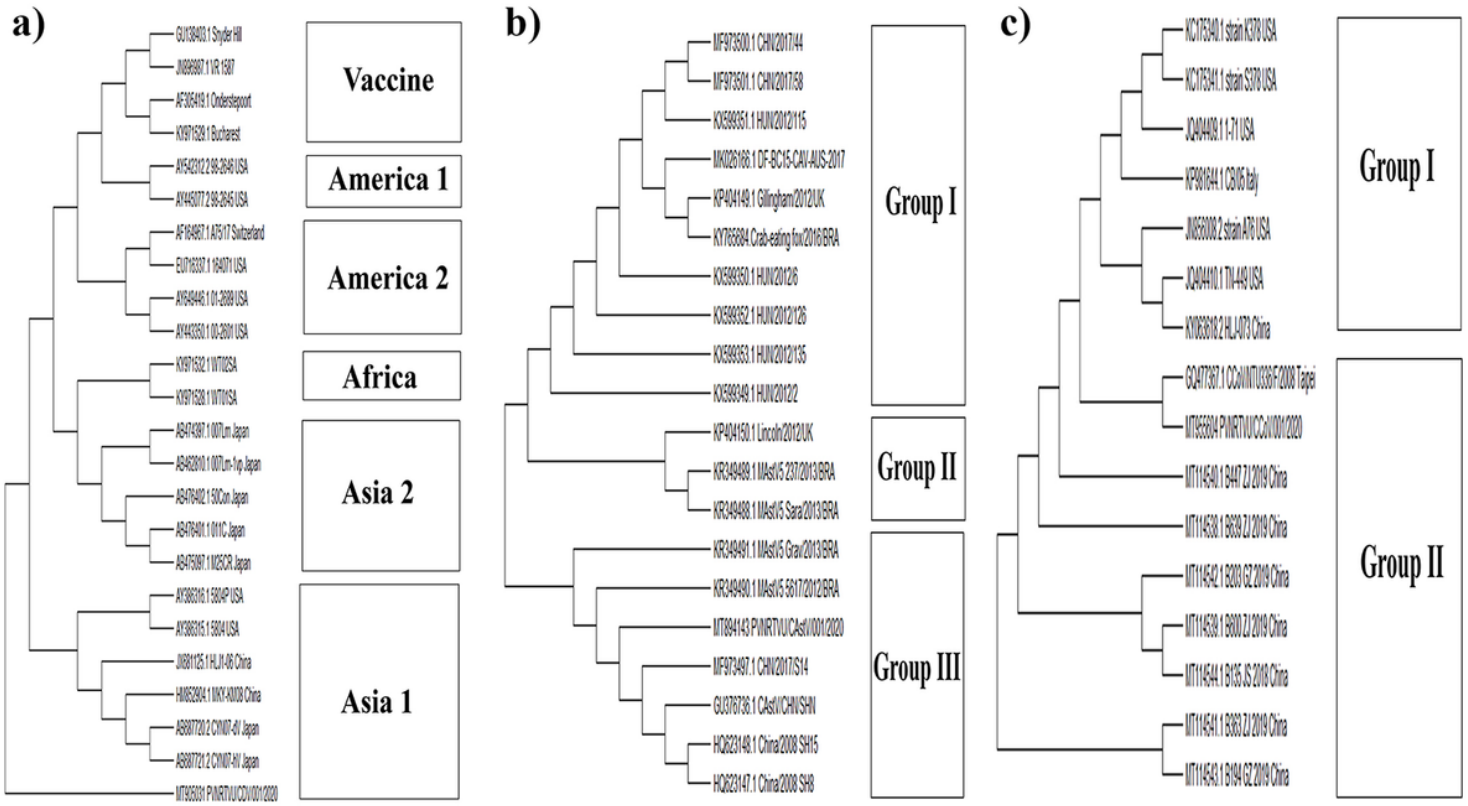

Figure 3

Phylogenetic analysis of the whole genome of current isolates against published sequences from NCBI database. The CDV isolates from current study clustered with Asia 1 lineage (a), CaAstV isolates from current study clustered with group III that consists of China and Brazil (b), CCoV isolates from current study clustered with group II that consists of China and Taipei isolates.

Supplementary Files

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- Supplementarydata.docx