MOLECULAR CHARACTERIZATION OF CANINE DISTEMPER VIRUS AND CANINE ASTRO VIRUS FROM CASES OF CANINE GASTROENTERITIS

BY

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CERTIFICATE

This is to certify that **Miss TALLAPALLY MOUNIKA (RVM/18-04)** has satisfactorily prosecuted the course of research and that the thesis entitled **"MOLECULAR CHARACTERIZATION OF CANINE DISTEMPER VIRUS AND CANINE ASTRO VIRUS FROM CASES OF CANINE GASTROENTERITIS"** submitted is the result of original work done and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by her for a degree of any University.

Date: 8/9/2021
Place: Hyderabad

(Dr. P. KALYANI)
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CERTIFICATE

This is to certify that the thesis entitled **"MOLECULAR CHARACTERIZATION OF CANINE DISTEMPER VIRUS AND CANINE ASTRO VIRUS FROM CASES OF CANINE GASTROENTERITIS"** submitted in partial fulfillment of the requirements for the degree of **Master of Veterinary Science** of **P. V. Narsimha Rao Telangana Veterinary University** is a record of *bona fide* research work carried out by **Ms. TALLAPALLY MOUNIKA (RVM/18-04) under** our guidance and supervision. The subject of thesis has been approved by the Student's Advisory Committee.

No part of the thesis has been submitted by the student for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of investigations have been duly acknowledged by the author.

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LIST OF ABBREVIATIONS

Abbreviation		Full form
%	:	Percent
>	:	Greater than
<	:	Less than
/	:	Per
µg	:	Microgram
µL	:	Microliter
µm	:	Micrometer
ºC	:	Degree Celsius
3’	:	3-prime
5’	:	5-prime
∞	:	Infinite
A	:	Adenosine
aa	:	Aminoacids
bp	:	Base pair
C	:	Cytosine
CO_2	:	Carbon dioxide
CPE	:	Cytopathic effect
dH_2O	:	Distilled water
DMEM	:	Dulbecco’s Modified Eagle Medium
DMSO	:	Dimethylsulphoxide
DNA	:	Deoxyribonucleic acid
dNTPs	:	Deoxynucleotide triphosphate
e.g.	:	Exempli gratia (for the sake of example)
EDTA	:	Ethylene diamine tetra acetic acid
et al	:	Et alia (and others)
EtBr	:	Ethidium bromide
F	:	Forward

FBS	:	Fetal bovine serum
Fig.	:	Figure
G	:	Guanosine
HCl	:	Hydrochloric acid
hr	:	Hour
IU	:	International unit
Kb	:	Kilobase pair
MDCK	:	Madin Darby canine kidney cell line
Min	:	Minute
mg	:	Milligram
mL	:	Milliliter
N	:	Normal
$NaHCO_3$	:	Sodium bicarbonate
NaOH	:	Sodium hydroxide
nm	:	Nanometer
No.	:	Number
PBS	:	Phosphate buffer saline
PCR	:	Polymerase chain reaction
pg	:	Pico gram
pH	:	Potential of hydrogen
Pmol	:	Pico mole
Psi	:	Pound force per square inch
R	:	Reverse
rpm	:	Revolutions per minute
sec	:	Seconds
T	:	Thymine
T_{25}	:	Total surface area 25 cm^2

T_{75}	:	Total surface area 75 cm^2
TAE	:	Tris acetate EDTA
T_m	:	Melting temperature

DECLARATION

I, **TALLAPALLY MOUNIKA (RVM/18-04)** hereby declare that the thesis entitled **"MOLECULAR CHARACTERIZATION OF CANINE DISTEMPER VIRUS AND CANINE ASTRO VIRUS FROM CASES OF CANINE GASTROENTERITIS"** submitted to P. V. Narsimha Rao Telangana Veterinary University for the degree of **MASTER OF VETERINARY SCIENCE** is a result of original research work done by me. It is further declared that the thesis or any part thereof has not been submitted for any other degree or diploma.

Date: 8/9/2021
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(TALLAPALLY MOUNIKA)

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ABSTRACT

Gastroenteritis refers to inflammation of the gastrointestinal tract resulting in vomitions and diarrhea. Viruses associated with enteric illnesses in dogs are an important cause of mortality. This study was majorly focused on viral enteritis caused by RNA viruses such as canine astrovirus (CAstV) and canine distemper virus (CDV).

The present study was targeted to isolate, characterize and to determine the molecular evolutionary dynamics of RNA viruses such as CAstV and CDV causing gastroenteritis in India.

A total of 150 fecal samples were collected from dogs exhibiting gastroenteritis, presented to various private clinics in Hyderabad and Teaching Veterinary Hospital, C.V.Sc, Hyderabad. These samples were subjected for molecular detection, characterisation, and isolation studies.

The faecal samples obtained from the suspected dogs were processed, mRNA was extracted, cDNA was synthesized and used as a template. All the samples were screened for CAstV and CDV by using Polymerase Chain Reaction (PCR). Six out of 150 samples detected positive for CAstV and five samples were positive for CDV. Two out of five cultured samples showed characteristic CPE in B-95a cells for CDV and were confirmed as CDV by PCR. All the six cultured samples showed a characteristic CPE in MDCK cells for CAstV, one was confirmed as CAstV.

Nucleotide sequencing of ORF-1b gene of CAstV PVNRTVU/2020/CAstV 01-04 (GenBank Acc. No. MT955600-03) showed 97-99% nucleotide identity with each other and clustered into Group III which consists of China and Brazil isolates on phylogenetic analysis.

Nucleotide sequencing of partial-H gene of CDV isolate PVNRTVU/2020/CDV (GenBank Acc. No. MW014320) with reference to vaccine strains and other sequences of CDV isolates from GenBank showed 91-95% identity. Multiple sequence alignment of partial-H gene sequence with reference to other sequences in NCBI database revealed that there is NdeI restriction site confirming them as wild type strains not the vaccine strain.

Further, the whole genome sequencing was done for CDV and CAstV for the first time in India. Upon phylogenetic analysis, the current CDV isolate clustered into Asia-I lineage and CAstV clustered into Group-III lineage.

From the present study, it may be concluded that there is an immediate need to emphasize on the development of the multivalent vaccine with the current circulating strains of CDV. The present study also revealed that CAstV have a significant role in canine gasteroenteritis, suggesting the need of extensive studies throughout the country on the prevalence of CAstV for effective control measures.

CHAPTER I

INTRODUCTION

Of all the domesticated animals, dogs have been the best companion animal of all ages. They are prone to several bacterial and viral diseases, most of which cause gastroenteritis. Gastroenteritis refers to inflammation of the gastrointestinal tract, meaning the stomach and the intestines. It is quite challenging for a veterinarian to determine the causative agent for diarrhea. The most probable reason is due to the diverse array of pathogenic agents that could be a cause of gastroenteritis including viruses, bacteria, or protozoans (Gizzi *et al.*, 2014). Reportedly viruses are the common cause with one or more viruses detected in 40–60% of gastroenteritis cases in dogs (Alves *et al*., 2018). Gastrointestinal disorders are frequently reported in companion animal clinics as leading to severe dehydration and death. This condition is often accompanied by abdominal pain, diarrhea and vomiting.

Viruses associated with enteric illnesses in dogs are an important cause of mortality in non-protected populations (Decaro and Buonavoglia, 2012). The two major causes of gastroenteritis of viral origin include Canine Distemper Virus (CDV) and Canine Astro Virus (CAstV). CDV infection results in gastrointestinal disorders, nervous and respiratory signs. CAstV has been reported to be associated with outbreaks of enteritis in puppies (Martella *et al.*, 2011).

CAstV belonging to the genus *Mammastrovirus* and family *Astroviridae* (ICTV, 2020) is a non-lipid enveloped, icosahedral, positive-sense, single-stranded RNA of 6.4–7.3 kb (Mendez and Arias, 2007), containing three open reading frames (ORFs), 5′ untranslated region (5′-UTR) and a 3′ poly-A tail (King *et al.*, 2011). ORF1a and ORF1b

located at the 5′ end of the genome encode non-structural proteins. ORF2 encodes a capsid protein, which is located at the 3′-terminal end (Martella *et al.*, 2011).

CAstV which is the causative agent of gastroenteritis in pet dogs was first identified in the early 1980s (Toffan *et al.,* 2009). However, it has been reported in the feces of dogs with and without diarrhea (Zhou *et al.*, 2017). Most of the studies show that the positive rate of CAstV in dogs with gastroenteritis is higher than that of dogs without clinical signs (Caddy and Goodfellow, 2015 and Zhou *et al.,* 2017), which indicates the role of CAstV in canine diarrhea. However, co-infection of CAstV with other viruses, such as Canine parvovirus (CPV), Canine coronavirus (CCoV) and CDV is ubiquitous (Martella *et al.,* 2011; Zhou *et al.,* 2017 and Li *et al*., 2018). Young pups (<12 months) are more susceptible to CAstV than adult dogs (>12 months). It has higher co-infection rate with other enteroviruses, such as CCoV and CPV-2, in young dogs compared with adult dogs (Zhou *et al.,* 2017).

Canine distemper (CD) a disease caused by CDV is an acute, highly infectious viral disease, characterized by rapid onset of severe leucopenia and loss of lymphocyte proliferation ability resulting in immunosuppression. It is distributed worldwide and results in high morbidity and mortality (Appel, 1987). The disease was first reported in Europe in 1790 (Appel and Gillespie, 1972).

CDV belongs to the genus *Morbillivirus* within the family *Paramyxoviridae* (ICTV, 2020) and order *Mononegavirales.* CDV is a single-stranded, negative-sense, non-segmented, enveloped RNA virus with a diameter of about 150-300 nm. The genome size of CDV is about 15.7 kb which encodes for six structural proteins; nucleocapsid (N),

matrix (M), fusion (F), haemagglutinin (H), polymerase (L) and phosphoprotein (P) (Messling *et al.*, 2001).

Morbillivirus is transmitted by aerosols, direct contact, and produce clinical signs, such as fever, serous nasal discharge, cough and gastrointestinal signs often complicated by secondary bacterial infections. Furthermore, the most notorious property of morbillivirus infection is establishing of severe transitory immunosuppression (Messling *et al*., 2003 and Nathanson and Griffin, 2007). The disease has been controlled by the use of attenuated live virus vaccines. However, several CDV vaccinated dogs also come down with CDV worldwide (Moller *et al.*, 1992). These findings suggest the difference in virulence between the wild-type CDV and vaccine strains (Harder and Osterhaus, 1997).

There are limited reports of whole genome sequences of CAstV and CDV from abroad (Caddy and Goodfellow, 2015 and Romanutti *et al.*, 2020) and there were no reports from India. The present study was hence taken up to genetically characterize geographically circulating CDV & CAstV isolates from cases of canine gastroenteritis in India during the period 2019-2020 and to unravel the molecular relationship with other published isolates of CDV and CAstV across the world.

Objectives of investigation

1. Detection of CDV and CAstV from fecal samples of canine gastroenteritis clinical cases.
2. Isolation and whole-genome characterisation of CDV.
3. Isolation and molecular characterisation of CAstV.

CHAPTER II

REVIEW AND LITERATURE

HISTORY

CAstV

CAstV was first identified in the early 1980s (Williams *et al.,* 1980 and Gough *et al.,* 1984) and it was characterised as a distinct *Mamastrovirus* species (ICTV, 2020). It is the causative agent of gastroenteritis in pet dogs (Toffan *et al.,* 2009). CAstV was first described in the USA, following the identification of star-shaped particles in diarrheic stools from a litter of beagle pups (Williams, 1980). Later on it has been identified in Italy, France, China, Korea and Brazil (Martella *et al.,* 2011; Zhu *et al.,* 2011; Grellet *et al.,* 2012; Castro *et al.,* 2013 and Choi *et al.,* 2014).

CDV

CDV is the causative agent of CD, an acute, highly infectious viral disease and the most important worldwide disease of domestic dogs. The disease was imported from South America to Europe around 1760 (Blancou, 2004). Edward Jenner first described the course and clinical signs of the disease in 1809 (Shell, 1990). CD was first described in Spain in 1791 and was first isolated by Carre. H, 1905 (Appel and Gillespie, 1972), hence the disease is still called Carre's disease and over time, it has been reported in a broad range of terrestrial (Summers *et al.,* 1994) and aquatic carnivores as well as in non-carnivore species (Forsyth *et al.,* 1998). Currently, at least 14 distinct genetic lineages of CDV are recognized worldwide, based on

sequence analysis of the *H gene*: Asia-1, Asia-2, Asia-3, Asia-4, America-1, America-2, America-1, South America-2 and South Africa-3 (Zhao *et al.*, 2010; Guo *et al.*, 2013; Radtanakatikanon *et al.*, 2013 and Espinal *et al.*, 2014).

ETIOLOGY AND HOST SUSCEPTIBILITY

CAstV

CAstV has been detected in multiple countries, in fecal samples from healthy or diarrheic dogs which demonstrates its global distribution (Toffan *et al.*, 2009; Grellet *et al.*, 2012 and Martella *et al.*, 2012). Astro viral infections were associated with gastroenteritis in most animal species, and human astroviruses are regarded as the second or third most common cause of viral diarrhoea in children (Mendez and Arias, 2007).

CDV

CDV is classified under the genus *Morbillivirus* within the *Paramyxoviridae* family (ICTV, 2020). CDV is closely related to other morbilliviruses such as measles virus (MV) of human and non-human primates; rinderpest virus (RPV) of cattle, pigs, goats, sheep, buffalos, elands, giraffes, kudus, and warthogs; peste des petite ruminants virus (PPRV) of goats, sheep, gazelles and ibexes (Dungworth, 1993 and Osterhaus *et al.*, 1995). CDV infects other mammals including species of the families Mustelidae (ferret, mink, skunk, weasel, badger, and marten), Procyonidae (raccoon,

coati and kinkajou), Ailuridae (lesser and giant pandas), Ursidae (bear), Viverridae (fossa, mongoose, civet, linsang, binturong, and genet), Hyaenidae (hyena) and Felidae (cheetah, lion, jaguar, margay, tiger, cat and ocelot) (Appel, 1987 and Dungworth, 1993).

VIRION STRUCTURE

Genome Organization of CAstV

CAstV genome is of 6.5-6.6 kb in length which constitutes three ORFs (ORF1a, ORF1b and ORF2) with a 5'-UTR, and 3'poly-A tail (King *et al.*, 2011). The schematic representation of the CAstV genome is shown in Fig 2.1.

ORF1a is located at the 5′ end of the genome which encodes non-structural proteins. ORF1b is 1530 nucleotide long and is predicted to encode for an RNA-dependent RNA polymerase (RdRp). The RdRp recognition site is located at positions 168–373 of the ORF1b amino acid sequence. Furthermore, three conserved motifs can be observed at positions 321 (GNPSG), 364 (YGDD) and 391 (FGMWVK) in the ORF1b amino acid sequence (Mihalov-Kovacs *et al.*, 2017).

The length of ORF2 is variable among CAstVs showing a range of 2472–2505 nucleotide (823–834 amino acids). The entire capsid protein precursor can be divided into three regions, region I (residues 1–446), region II (residues 447–730) and region III (residue 731–end).

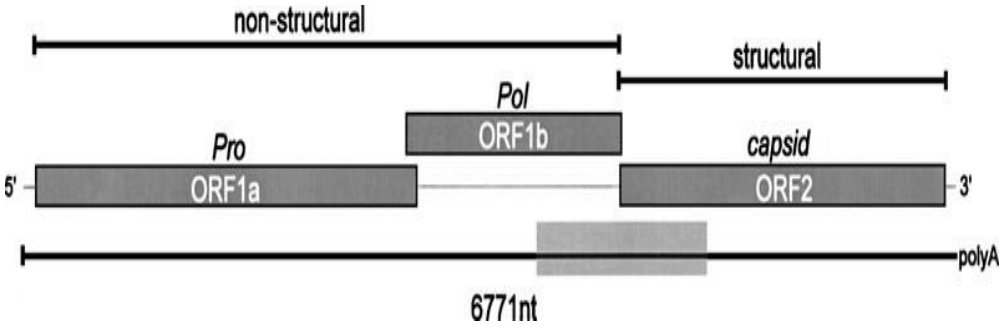


Fig 2.1: Schematic representation of the genome organization of CAstV (Li *et al.*, 2018).
Map of genomic RNA from 5'- 3', 5'-UTR, ORF1a, ORF1b and ORF2 with a 3'poly-A tail.

Genome Organization of CDV

CDV is a single-stranded, negative-sense (3′-5′), non-segmented, lipoprotein enveloped RNA virus with a diameter of about 150-300 nm. The genome of CDV is about 15.7 kb, which consists of six genes encoding six structural proteins (Murphy *et al.*, 1999). The organization of the major gene codes in the CDV genome is 3′-N-P-M-F-H-L-5′, each separated by UTRs (Griffin, 2001). The schematic representation of the CDV genome is shown in Fig 2.2**.**

CDV Encoded Proteins: Six genes encode six structural proteins; two glycoproteins (H and F protein), a single envelope-associated M protein, two transcriptase associated proteins (P, L) and the N protein that encapsidates the viral RNA (Regenmortel *et al.*, 2000). The non-structural protein (C) and (V) were produced by an alternative open reading frame in the P gene (Lamb and Kolakofsky, 2001).

The "H" glycoprotein is responsible for the viral attachment to the host cell (Murphy *et al.*, 1999). The H gene is one of the most variable genes in CDV and has

been used to investigate the genetic relationships among the various strains (Gamiz *et al.*, 2011). The F protein is a glycoprotein that is essential for mediating fusion between the viral particle and the host cell membrane. The F protein provides the virus with the necessary mechanism to move from one host cell to another (Murphy *et al.*, 1999). The M protein is important for the entry of the virus into a susceptible cell and this protein plays an important role in the assembly of new viral particles (Murphy *et al.*, 1999). The P and the L proteins form a functional polymerase complex and are responsible for the replication of viral RNA. The nucleocapsid (NP) protein is responsible for the protection of the viral RNA (Murphy *et al.*, 1999).

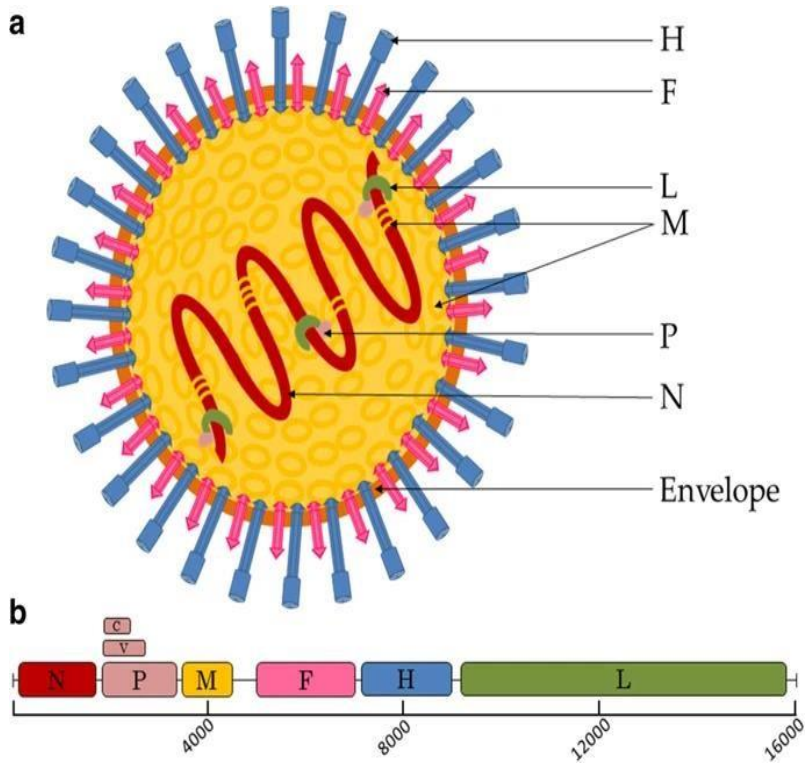


Fig 2.2: Schematic representation of the ultrastructural morphology and genetic features of CDV (Rendon-Marin *et al.*, 2019).
a). Schematic diagram of CDV particle in cross-section N: nucleocapsid, P: phosphoprotein, M: matrix protein, F: fusion protein, H: hemagglutinin, L: large polymerase protein. **b).** Map of genomic RNA (3′ to 5′) of CDV. Each box represents a separately encoded mRNA; multiple distinct ORFs within a single mRNA are indicated in overlapping boxes on P.

VIRION PROPERTIES

CAstV

The virions of CAstV shed in feces are 28–30 nm in diameter, spherical in shape and non-enveloped. The virions derived from cell culture are up to 41 nm in diameter, with well-defined surface spikes. The virions are resistant to pH 3, heating at 50ºC for 1 hr or 60ºC for 5 min, chloroform, lipid solvents and non-ionic, anionic, and zwitterionic detergents (Kurtz and Lee, 1987).

CDV

CDV is extremely sensitive to UV radiation, heat desiccation (50-60 ºC), oxidizing agents, detergents and lipid solvents. At room temperature the virus is short-lived, surviving between 20 min to 2 hrs in tissues and exudates. Virus can survive for several days at temperatures below zero if protected by organic material (Greene *et al.*, 1984). The virus remains viable between pH 4.5-5, and it is susceptible to organic solvents like ether, chloroform, dilute (< 5) formalin solution, phenol (0.75) and quaternary ammonium disinfectant (0.3). CDV in the infected tissues was shown to retain infectivity for days at 25°C and weeks at 2-4°C (Shen and Gorham, 1980). CDV can survive at -65ºC for at least 7 years (Greene and Appel, 1998).

TRANSMISSION

CAstV

CAstV has been detected exclusively in the stools and the intestinal content of pups. CAstV can be detected by RT-PCR atleast upto 10 days in a 3-month-old pup hospitalized with watery diarrhea and severe dehydration. Viral shedding continues for 8 days after the pup recovered from the disease (Martella *et al.*, 2011). Prolonged viral shedding after acute infection and resistance in the environment (Abad *et al.*, 1994) could be factors facilitating virus diffusion in a susceptible population.

CDV

The most common source of infection of CDV is direct contact between the susceptible dog and an infected dogs or wildlife. CDV is commonly transmitted by aerosol or droplet exposure originating from respiratory exudates; however, urine and other secretions also contain an infectious virus (Greene and Appel, 1998). Its incubation period ranges from about 1 week to 1 month. CD is highly contagious, and viral shedding may follow infection for 60–90 days (Appel, 1987). Transplacental infection has been documented in domestic dogs (Krakowka *et al.*, 1977).

EPIDEMIOLOGY

CAstV Status in Abroad

CAstV was first identified in the stools of beagle puppies with diarrhea in the United States in 1980 (Williams, 1980). However, these reports were based on electron microscopy descriptions only, thus the correct and complete identification of the small virus particle detection was not possible. The occurrence of astrovirus infection in dogs was only recently confirmed by RT-PCR detection and further genetic characterization by sequencing (Tooffan *et al.,* 2009).

In 2011, CAstV has been successfully isolated in cell culture for the first time (Martella *et al.,* 2011). A study on the prevalence of CAstV in Shanghai, China, suggested that 12% (22/183) of the puppies showing clinical signs of diarrhea were positive for astrovirus by RT-PCR as compared to none of the 138 healthy controls (Zhu *et al.,* 2011).

A study conducted in Italy, detected CAstV in 24.5% of 110 stool samples collected from symptomatic dogs and 9.3% of 75 stool samples from asymptomatic animals (Martella *et al.,* 2011). In the same study, serologic assay indicated that 59% of 54 dogs aged >3 months surveyed in Italy presented specific CAstV antibodies (Martella *et al.,* 2011).

A similar prevalence based on viral RNA detection was found in France where 20.9% (66/316) of the puppies screened were CAstV-positive by RT-PCR (Grellet *et al.,* 2012).

Stool samples were collected from pet dogs in the UK with and without gastroenteritis, and samples were screened for CAstV by qPCR. Four CAstV positive samples were identified from dogs with gastroenteritis (4/67, 6.0%), whereas no

samples from healthy dogs were positive. Sequencing of the capsid sequences from the four CAstV strains found significant genetic heterogeneity, with only 80% amino acid identity between strains (Caddy and Goodfellow, 2015).

A study in Japan collected fecal samples from diarrheic and asymptomatic dogs and examined the feces for the presence of CAstV by RT-PCR with primers based on a conserved region of the ORF1b gene. The ORF1b gene of CAstV was not detected in the 42 dogs without clinical illness but was present in three pups out of the 31 dogs with diarrhea symptoms. Based on the full-length capsid protein, the CAstV KU-D4-12 strain that detected here shared high homology with the novel virulent CAstV VM-2011 strain (Takano *et al.,* 2015).

A study to investigate CAstV infection in southwest China revealed that 42/107 diarrheic samples (39.3 %) were positive for CAstV by RT-PCR, and 41/42 samples showed co-infection with CCoV, CPV-2 and CDV. Phylogenetic analysis based on 26 CAstV partial ORF1a and ORF1b sequences revealed that most CAstV strains showed unique evolutionary features (Li *et al.,* 2018).

As per literature cited, no reports were found on CAstV in India.

Status of CD in Abroad

The modified live vaccine developed in late 1950 was used to control CD successfully. The negligence of vaccination, leading to poor vaccine coverage and herd immunity, was an obvious reason for outbreaks at the population level. Although the use of modified live vaccines has significantly reduced the incidence of CD, the outbreaks of CD were reported in vaccinated dogs (Glardon and Stockli, 1985 and

Adelus-Neveu *et al.,* 1991).

During the 1980s, outbreaks of distemper occurred in dogs throughout Europe (Glardon and Stockli, 1985 and Adelus-Neveu *et al.,* 1991) and in 1990; the disease reappeared in Finland after an absence of 16 years.

The epidemiological studies suggested that CD as an endemic in Brazil mostly in the urban canine population, with frequencies of RT-PCR positive strains ranging from 47.1% to 66.5% dogs with signs of distemper (Gebara *et al.,* 2004).

Distemper was detected in wild raccoons on the grounds of a large zoo near Chicago (USA) in the years 1998, 2000, and 2001 (Lednicky *et al.,* 2004). Between 2003 and 2004, an increase in CDV circulation was detected in Argentina, including in vaccinated animals (Calderon *et al.,* 2007).

Due to vaccination, the outbreak of CD disease has been controlled. However, the incidence of CDV in canines and other animal populations throughout China has been increased (Perpinan *et al.,* 2008). Outbreaks of CD have also been reported in vaccinated dogs in Italy as well as in other countries (Balboni *et al.,* 2014).

CD Status in India

In India, CDV infections are quite common and serological surveys using dot enzyme-linked immunosorbent assay (ELISA) has been done. Out of the 160 samples tested by Dot-ELISA, 112 (70%) were positive for CD. This study suggested that vaccination followed by regular annual boosters against distemper would effectively protect the dogs from the disease (Latha *et al.,* 2007)

Ramanathan *et al.* (2007) found 49 lions positive for CD out of 56 lions (87.5%) in a serosurvey on captive Asiatic lions from Western India. The evidence for current or past exposure to CDV was found in 90.7% of an individual from a survey of free dog population at Great Indian Bustard Sanctuary, Maharashtra, India (Vanak *et al.*, 2007). Srivastav and Nigam, (2010) reported that CD was a major threat to the Asiatic wild dog population in India.

Pawar *et al.* (2011) reported the isolation of Indian CDV from B95a cells and sequence analysis of N gene. Swati *et al.* (2015) sequenced and characterized the full-length F, P and M genes of wild type CDV from India. They mainly focused on the cloning, sequencing and the phylogenetic analysis of an Indian CDV strain; compared against the reference CDV's available from GenBank and the commercial CDV vaccine strains.

Ashmi *et al.* (2017) detected CDV in 19(21%) dogs out of 90 CDV suspected dogs by RT-PCR and 21(23%) samples by nested PCR. Phylogenetic analysis of partial H and F gene nucleotide sequences revealed that the field CD viruses of that study were distinct and varied from the vaccine strain.

Mourya *et al.* (2019) detected CDV in 68 lions and 6 leopards by RT-PCR. Whole genome sequence analysis demonstrated that the virus strain is similar to the proposed Asia-I.

Abirami *et al.* (2020) collected ocular and nasal swabs were collected from 40 dogs suspected for CD from Puducherry state and detected CDV in 15 (37.5%) out of 40 ocular/ nasal swabs by RT-PCR targeting the N gene. The CDV sequences were closer to the CDVs of Africa - 2 lineage than the other Asian lineages

DIAGNOSIS

Diagnosis of CAstV

Antibodies specific for CAstV (virus Bari/08/ITA) have been detected by an indirect immunofluorescence assay in 32 of 54 (59.0%) serum samples**.** The majority (14 of 22, 63.6%) of the serum samples tested negative were from pups aged less than 3 months. Only 3 of 32 (9.4%) positive sera were from dogs aged less than 3 months. This age-related pattern would be consistent with the fact that by 2 to 3 months of age, pups are susceptible to infectious agents, as maternally derived immunity tends to wane (Pollock and Carmichael, 1982).

CAstV has been detected on RT-PCR in 22 of 183 (12.02%) dogs with enteric signs but in 0 of 138 asymptomatic dogs (0%) upon a survey in Shangai, China, in 2008. The 3' end portion of the genome of a Chinese virus was sequenced and found to be distantly related (76.9% to 78.3% nucleotide identity in the full-length ORF2) to other CAstV, providing evidence for genetic heterogeneity in CAstVs (Zhu *et al.*, 2011).

Martella *et al*. (2011) detected CAstV, strain Bari/08/ITA from a pup with gastroenteric signs and the virus was isolated in cell culture and characterized molecularly. In the full-length capsid protein, the virus displayed genetic similarities (83.5 % amino acid identity) to another CAstV strain, although a high rate of variation occurred in the hypervariable domain, which was related to CAstV antigen specificity. CAstVs were detected in 24.5% of young pups with gastroenteritis, either alone or in mixed infections with other canine pathogens. In contrast, CAstV was detected in only 9.3% of asymptomatic pups. These findings indicate that CAstV is common in dogs and may suggest a possible role as canine enteric pathogens.

Choi *et al.* (2014) examined fecal samples from 91 Korean dogs suffering from diarrhea. CAstV was identified in 2 (2.1%) dogs. Nucleotide sequence analysis coupled with phylogenetic analysis using the neighbour-joining method showed that CAstV clustered into four genetically diverse groups. The results suggest that the Korean strain of CAstV was closely related to strains isolated in Europe.

Caddy and Goodfellow (2015) collected stool samples from pet dogs in the UK with and without gastroenteritis and samples were screened for CAstV by qPCR. Four CAstV positive samples were identified from dogs with gastroenteritis (4/67, 6.0%), whereas no samples from healthy dogs were positive ($p < 0.001$). The capsid sequences from the four CAstV strains shown significant genetic heterogeneity, with only 80% amino acid identity between strains. The full genome sequence of two UK CAstV strains confirmed that CAstV conforms to the classic genome organization of other astroviruses with ORF1a and ORF1b separated by a frameshift and ORF2 encoding the capsid protein.

Lizasoain *et al.* (2015) collected 20 domestic sewage samples in two cities of Uruguay from dogs. Four samples were characterized as CAstV. Upon phylogenetic analysis, CAstV clustered with strains detected in Italy 2008 and Brazil 2012.

Zhou *et al.* (2017) collected and tested 253 rectal swabs from pet dogs of which 64 samples (25.3%) tested positive for CAstV with diarrhea and 15 samples (5.9%) CAstV positive without any clinical signs. Phylogenetic analysis of 39 partial ORF1b sequences from these samples revealed that they are similar to CAstV that can be subdivided into three genetic lineages. Out of the 39 isolates sequenced, 16 isolates were Mamastrovirus 5/ CAstV lineage and the remaining 23 isolates displayed higher similarities with known porcine astrovirus 5 and 2.

Alves *et al.* (2018) investigated the presence of CAstV in 269 dog fecal samples by RT-PCR. CAstV was detected in 26% (71/269) of the samples. Phylogenetic analysis based on the ORF2 amino acid sequences shows they were closely related to Chinese samples.

Li *et al.* (2018) collected 107 fecal samples from domestic dogs with diarrhea. Forty-two diarrhoeic samples (39.3%) were positive for CAstV by RT-PCR. Phylogenetic analysis based on 26 CAstV partial ORF1a and ORF1b sequences revealed that most CAstV strains showed unique evolutionary features, suggesting that this strain might be a novel genotype.

Bhatta *et al.* (2019) investigated the possible infectious causes of gastroenteritis in puppies from a dog kennel in Victoria, Australia. The near-complete genome of CAstV was found to be 94.7% identical with a CAstV detected in the United Kingdom in 2012. The phylogenetic analysis of the capsid gene found similarities to those of CAstV identified in Italy in 2005 and the UK and Hungary in 2012, but distant from that of a CAstV previously identified in Australia in 2012. Thus, different serotypes of CAstV are likely circulating in Australia.

Zhang *et al.* (2020) found 14 (13.2%) and 7 (3.35%) CAstV positive samples from pet dogs with and without diarrhea, respectively. Phylogenetic analysis of the *ORF-2* gene revealed 4 major genetic lineages. In particular, the genetic lineage 4 might have evolved from a recombinant virus from lineage 2 and lineage 3. The strains clustered with lineages 2, 3, and 4 in contrast with other Chinese strains identified previously that clustered with lineages 2 and 4.

Diagnosis of CDV

CDV was readily isolated at a high rate with marked CPE in B95a cells, from the peripheral blood leukocytes, cerebrospinal fluid cells and brains of dogs. The difference in a type of CPE, i.e., syncytium type and round cell one, among the virus isolates indicates the presence of heterogeneity of virus populations in prevalent CDV. This cell system was expected to be useful for ecological studies on CDV in the field (Kai *et al.*, 1993).

Frisk *et al.* (1999) detected CDV nucleoprotein (NP) RNA in serum, whole blood, and cerebrospinal fluid (CSF) samples from 38 dogs clinically suspected of distemper. They exhibited greater homology to the Rockborn (97 to 99%) than to the Onderstepoort (95 to 96%) CDV strain.

Based on H gene sequences, Hashimoto *et al.* (2001) identified two distinct hemagglutinin genotypes in CDV circulating among dogs since 1991 in Japan. One is the native and numerically predominant KDK-1 genotype, while the other is the 98-002 genotype that may have evolved in the far East beyond the border between Japan and the Republic of Korea.

Martella *et al.* (2002) sequenced and phylogenetically analyzed the fragments of the genes encoding the H and N proteins of CDV like virus affecting red fox (*Vulpes vulpes*) and reported that all the Italian CDVs can be included in the H European genotype.

Lednicky *et al.* (2004) used tissue samples from raccoons with histologic evidence of distemper and used Vero, MDCK and MV 1 Lu cells for virus isolation. Among these cell lines, Vero cells showed the most obvious CPE.

The phylogenetic analysis of complete F and H genes and partial P gene sequence by Pardo *et al.* (2005) demonstrated three genetically distinct CDV strains from seven clinical samples of dogs in Columbia. These isolates were distant from those previously found in North America and most closely related to the isolates previously described in either Asia or Europe.

Martella *et al*. (2006) studied the H gene obtained from field strains of clinical specimens of Italian dogs. The phylogenetic analysis has shown a homogeneous group of CDV strains were widespread in Italian dogs, all of which were included in the European lineage.

Martella *et al.* (2007) demonstrated to distinguish field isolates from vaccine strains by Restriction fragment length polymorphism (RFLP) analysis of 829 bp fragment of the N gene of CDV using BamHI.

Calderon *et al*. (2007) amplified a fragment of the H gene from 24 (32.9%) samples of 73 nucleocapsid protein-RNA-positive clinical specimens. Phylogenetic analysis of the partial hemagglutinin protein sequences has shown a close cluster of local strains that are distinct from vaccine strains and other wild-type foreign CDV strains. One of the local strains, Arg 23, branched out of the root of the Argentine clade, close to the European strains, suggesting that two different pathogenic CDV genotypes were circulated in Argentina with one of them predominant.

Based on the H gene sequences Kapil *et al.* (2008) reported that the CDV isolates from three states (California, Missouri, and Oklahoma) formed two CDV genetic groups: group I (major; six of seven isolates) consisted of CDV isolates closely related to the European wildlife lineage of CDV, and group II (minor; one of seven isolates) was genetically related to the Arctic-like lineage of CDV. However, both CDV groups were genetically different from the current vaccine strains that

belonged to the American-1 lineage of the old CDV isolates.

An *et al.* (2008) sequenced the H genes from four CDV isolates obtained from three dogs and a marten in Korea. Phylogenetic analysis revealed eight clades designated as EU1, EU2, EU3, NA1, NA2, Asia 1, Asia 2 and Vaccine.

Muthiah *et al.* (2008) isolated CDV in MDCK and Vero cell lines. The samples were screened by RT-PCR followed by nested PCR. Two out of 25 field samples and three out of 5 cultured samples were found to be positive for CDV indicating an increase in the sensitivity by about 40% when samples were put for isolation on cell culture.

CDV was isolated in two 4-months old autopsied dogs in Vero cells and Vero SLAM cells by Lan *et al.* (2009) for the first time in Vietnam. The molecular analysis showed both new isolates of CDV were joined to the group of a classic type that is far from the Asia 1 and Asia 2 groups.

Chan *et al.* (2009) noticed the sequence variations in the H gene from the field CDV strain that had previously been implicated in the increasing incidence of CD in Taiwan.

Woma *et al.* (2010) isolated CDV from clinical samples and amplified the H gene from cell culture isolates. Based on the sequences of clinical isolates and vaccine strains, they found a novel CDV lineage existed in South Africa.

Zhao *et al.* (2010) sequenced the H gene of 28 CDV strains from both vaccinated and unvaccinated foxes, raccoon's dogs and minks from different geographical areas of China and concluded that at least three different CDV genotypes, distantly related to the vaccine strain were circulated in breeding foxes, raccoon, dogs and minks.

Pawar *et al.* (2011) sequenced the partial N gene of RT PCR positive samples and local vaccine virus. They reported that the Ind/Andaman 01/07 virus was highly divergent from the rest of the CDV isolates and the vaccine strain.

Guo *et al.* (2013) phylogenetically analyzed the H gene from eight CDV isolates obtained from seven raccoon dogs (*Nyctereutesprocyonoides*) and a giant panda (*Ailuropodamelanoleuca*) in China. They noticed the partial H gene was distinct from vaccine strains and other wild-type foreign CDV strains and all the CDV strains were characterized as Asia-1 genotype and were highly similar to each other (91.5-99.8% and 94.4-99.8%nucleotide and amino acid identity).

Maria *et al.* (2014) sequenced the full-length H gene of 15 wild-type CDV strains circulating in domestic dog populations from the Aburra Valley, Colombia. The phylogenetic analysis revealed that the Colombian wild-type viruses formed a distinct monophyletic cluster separated from the previously identified wild-type and vaccine lineages, suggesting that a novel genetic variant, quite different from vaccines and other lineages, circulated among dog populations in the Aburra Valley.

Budaszewski*et al.* (2014) collected a total of 386 rectal swabs from dogs with or without clinical signs suggestive of CDV infection in seven Brazilian states: 155 out of 386 animals were found to be CDV positive by RT-PCR. Samples were further screened by H gene fragment amplification; 13 of them had the complete H gene amplified. Upon sequence and phylogenetic analysis, all but one of the wild-type CDVs from Brazil clustered with strains from Uruguay, Argentina and European countries, within the South America-I/Europe genotype.

Castanheira *et al*. (2014) collected 53 rectal swabs from stray dogs. All rectal swabs were analyzed for the presence of CDV by quantitative PCR methods and 6 samples were detected as CDV.

Swati *et al.* (2015) collected ocular and nasal swabs from a total of 50 CD suspected dogs. The virus is successfully isolated from only one sample in MDCK cells after lymphocyte culture. Partial H and L gene sequences of one of the CDV isolates made a distinct clade in the phylogenetic tree, which was separated from commercial CDV vaccine groups.

Aarthi *et al.* (2015) amplified the F and H gene regions of CDV using RT-PCR. The partial gene was sequenced to compare the genetic variation among the field isolate and vaccine strain. The phylogenetic analysis suggested that CDV is prone to evolution and a different CDV lineage present in Tamil Nadu. Variations in the amino acid site of the F and H gene regions indicated the need for the development of new candidate vaccines in Tamil Nadu.

Nguyen *et al.* (2016) detected the H- gene in five domestic Vietnamese dogs with diarrhea, and two CDVs were successfully isolated from dogs positive for the H gene. The phylogenetic analysis showed all Vietnamese CDVs belonged to the Asia-1 genotype and homologous to those of Chinese CDVs (98.4% to 99.3% identity).

Cortez *et al.* (2017) phylogenetically demonstrated that the Brazilian CDV strains were genetically related to the circulating CDV strains in Uruguay, Argentina, and Europe. The degree of genetic divergence between wild Brazilian CDV strains and vaccine strains suggests the possibility of vaccine failures and consequently the occurrence of CD outbreaks.

Ashmi *et al.* (2017) collected 90 clinical samples from CDV suspected dogs, 19 samples (21%) were found positive by RT-PCR and 21 samples (23%) by nested PCR. When the N gene-positive cDNA was subjected to PCR amplification of partial H and F gene sequences, 2 H and F gene-positive amplifications were obtained. Phylogenetic analysis of partial H and F gene sequences revealed that the field CD

viruses of this study were distinct and varied from the vaccine strain. Out of 21 positive samples, none of them produced CPE even after 3 passages in Vero cells expressing SLAM and MDCK cell lines.

Chen *et al.* (2018) amplified the H gene from 15 samples out of 58 positive samples. The fifteen amplified H gene fragments were compared to CDV reference strains obtained worldwide. The phylogenetic analysis revealed that out of 15 strains, 9 clustered with the Asia-1 strains from China. Two strains were closely related to synderhill vaccine strain and four strains were closely related to the Ondersteport vaccine strain.

Maganga *et al.* (2018) reported an outbreak of 18 cases of CD that occurred in 2015 in a German shepherd dog population in northwestern Gabon. Phylogenetic analysis of whole-genome revealed that Gabonese CDV strain clustered with European strains belonging to the Europe genotype.

Piewbang *et al.* (2019) reported the complete genome sequences of eight CDV viruses from domestic dogs in Thailand. Most of the identified CDV strains (CDV1-3, -5, -8 TH/2014) clustered as a novel Asia-4 lineage, while the CDV4, -6, -7 TH/2014 belonged to the Asia-1 lineage. Recombination analysis revealed that the CDV4 TH/2014 is a putative recombinant virus from the Asia-1 and America-2 parent viruses.

Romanutti *et al.* (2020) isolated wild-type CDV strain (Arg24) from sick vaccinated dogs. This strain produced a strong cytopathic effect in Vero SLAM cells. The genome was completely sequenced using Illumina technology. The hemagglutinin gene, which was the target for genetic characterization, showed four additional potential glycosylation sites, concerning the Onderstepoort.

Yang *et al.* (2020) isolated CDV from 17 samples in Vero/dSLAM. The nucleotide and amino acid sequences of the H gene of isolated (CD1901) strain were compared with those of other CDV strains. The CD1901 strain belonged to Asia 1 group and had the highest similarity (99.9%) with the BA134 strain, which was isolated in China in 2008.

CHAPTER III

MATERIALS AND METHODS

GENERAL LABORATORY MATERIALS

Glassware

Neutral glassware of Borosil and Schott Duran make were used throughout the study.

Plasticware

Tissue culture flasks T-75 cm², T-25 cm² procured from 'TPP' were used in cell culture study. Disposable pipettes (2 mL, 5 mL, 10 mL) procured from 'Corning' were used. 'Axygen' and 'Tarsons' centrifuge tubes (15 mL), microfuge tubes (2 mL, 1.5 mL), PCR tubes (0.2 mL) and micropipette tips (1 mL, 200 µL, 10 µL) were used in this study.

Sterilization of Labware

Labware were scrubbed with neutral detergent (Labolene) and washed thoroughly under running tap water until all the traces of soap were removed. Later, they were soaked and rinsed thrice with distilled water. Finally, they were rinsed with double distilled water and kept inverted for air drying. Dried glassware were packed and sterilized in hot air oven at 160^{o}C for one and a half hr. Dried plastic ware like bottle screw caps, filter assembly along with filter membrane were packed and sterilized by

autoclaving at 121ºC/15 psi/15 min. They were also decontaminated thoroughly using 70% ethyl alcohol and UV light wherever necessary.

CHEMICALS AND REAGENTS

Chemicals used for cell culture work in the present experiment were of 'AnalaR' or 'ExcelaR' grade from Gibco, HiMedia, Qualigens, Sisco Research Laboratories (Mumbai, India) and Sigma Aldrich (St. Louis, USA). Molecular grade reagents were used for the preparation of all solutions and buffers. The reagents and labware were availed from suppliers including Merck (Mumbai), Qualigens, Sigma Aldrich (Hyderabad) and Fischer scientific. Chemicals used for molecular work in the present study were of molecular grade from TaKaRa, HiMedia, New England BioLabs, Sigma (USA) and Life Technologies.

All aqueous solutions were prepared using double-distilled water. Where necessary, solutions were sterilized by autoclaving at 121ºC, 15 psi for 15 min or by membrane filtration using 0.22 μm membrane filter (Catalogue No.GVWP04700; Millipore).

Growth Medium

Growth medium was prepared by supplementing DMEM with 10% FBS (Ref.No. RM1112-500 mL; HiMedia).

Maintenance Medium

Maintenance medium was prepared by supplementing DMEM with 1% FBS. Maintenance medium was used during the maintenance period of the cell layer and not for subculture. During maintenance phase, the cells will survive but the rate of division is slow. Hence only 1% FBS was used.

Freezing Medium

Freezing medium was prepared by adding 10% cryoprotectant, Dimethyl sulphoxide (042982; SRL) and 20% FBS in MEM. This solution was mixed by gently swirling. The prepared solution was sterilized by filtering through 0.22 µm syringe filter.

CELL LINES

Epstein-Barr virus transformed marmoset B lymphoblastoid cell line (B95a) cell lines were used for the present research purpose. B95a cell line was procured from Translational Research Platform for Veterinary Biologicals (TRPVB), TANUVAS, Chennai, Tamil Nadu.

Madin-Darby Canine Kidney (MDCK) cell line was procured from National Centre for Cell Science, Pune (India) and was maintained in the Department of Veterinary Biotechnology, College of Veterinary Science, Rajendranagar, Hyderabad.

Cryopreservation of Cells

Freezing of Cells: Cell layer at late log phase of growth was processed by decanting the medium and washing monolayer with 2 mL of 1X PBS gently. After decanting PBS, 1 mL of 0.2% trypsin was added ensuring that the monolayer is completely covered. All but a few drops of the trypsin were removed gently by tilting the flask. Then the flask was incubated at 37^{o}C, until the cells round up. 2 mL of growth medium was added immediately and the cells were dispersed by repeated pipetting. Cell count was carried out by trypan blue dye exclusion assay. Then cell pellet was obtained by centrifugation at 4000rpm/10min.

Cells at the concentration of 2 X 10^{6}cells/mL were suspended in the freezing medium and aliquoted into cryovials. Then the sealed vials were placed in pre-cooled freezing container containing isopropanol and allowed to freeze at the rate of 1°C/min by placing at -70°C for 3 hrs. Then the vials were immediately transferred into liquid nitrogen container (-196°C).

Thawing of Cryopreserved Cells: Vial containing cryopreserved cells, was immediately transferred from liquid nitrogen container to water bath at 40°C for 5 min allowing rapid thawing. Then cell pellet was obtained by centrifugation at 500 rpm/10 min. The freezing medium was discarded and cells were washed with plain medium at 3000 rpm/10 min. Fresh growth medium was added to the obtained cell pellet. Then based on the cell viability count by trypan blue assay, seeding of cell culture flask was done.

3.3.3 Subculture of Cells

T_{25}flask with healthy confluent monolayer was processed by decanting the medium and washing monolayer with 2 mL of 1X PBS gently. After decanting PBS, 1 mL of 0.2% trypsin was added ensuring that the monolayer is completely covered. All but a few drops of the trypsin were removed gently by tilting the flask. Then the flask was incubated at 37ºC until the cells round-up (5 min). To it, 3 mL of growth medium was added immediately and the cells were dispersed by repeating pipetting.

Cell count was carried under an inverted microscope with the hemocytometer by trypan blue dye exclusion assay. Later, cells were distributed into T_{25} flasks at a seeding concentration of 2 X 10^6cells/mL. This was done by diluting the cells to the total volume required and distributing that volume among several flasks. Each flask has 5-7mL of cell suspension so that the whole surface of the flask was covered. The flasks were incubated in an incubator at 37ºC with 5% CO_2 and cells were observed every 24 hrs to check for the formation of a confluent monolayer. The monolayer after attaining 70% confluency was used for virus infection.

TRYPAN BLUE EXCLUSION ASSAY

The cell suspension was mixed thoroughly to take 50 µL of cell sample. This cell sample was added to 450 µL of 0.4% Trypan blue solution and mixed gently. Neubauer chamber with glass cover was placed on the microscope stage. Then 20 µL of the mixture was loaded into the chamber of hemocytometer slide, by capillary action. Live cells were counted by 10X objective, in the central 1mm² area of grid.

The cell concentration was calculated using the formula given below.

$c = n \times 10^4 \times$ dilution factor Dilution factor = 10

c - Cell concentration (cells /mL)

n - Average number of cells in four $1mm^2$ area

CLINICAL SAMPLES

Collection of Samples

A total of 150 fecal samples/rectal swabs (Table 3.1)were collected from dogs exhibiting clinical signs of CDV such as (enteritis, gastritis, nasal discharges, conjunctivitis, pyrexia, etc.) from Teaching Veterinary Hospital, College of Veterinary Science, Rajendranagar, Hyderabad and various private clinics in Hyderabad**.** These clinical samples were used in the present study.

Table 3.1: Details of fecal samples used in this study

Sample no.	Sample ID	Breed	Age (months)	Vaccination status
1	F-1	Pug	8	Yes
2	F-2	Labrador	2	Yes
3	F-3	Labrador	13	Yes
4	F-4	Pomeranian	6	No
5	F-5	German Shepherd	9	Yes
6	F-6	Mongrel	5	No
7	F-7	Labrador	4	No
8	F-8	Mongrel	3.5	No
9	F-9	Shih Tzu	4	Yes
10	F-10	Mongrel	7	-
11	F-11	German Shepherd	4	Yes
12	F-12	Labrador	4	Yes
13	F-13	German Shepherd	6	No
14	F-14	Mongrel	3	No
15	F-15	Dacshund	2	Yes
16	F-16	German Shepherd	3	No
17	F-17	Pug	4	Yes
18	F-18	Mongrel	2	No
19	F-19	Great dane	4	No
20	F-20	German Shepherd	2	No
21	F-21	Pomeranian	12	Yes
22	F-22	Labrador	2	No
23	F-23	Labrador	1	No
24	F-24	Pomeranian	12	No

Table 1 (Cont.).

Sample no.	Sample ID	Breed	Age (months)	Vaccination status
25	F-25	Bullmastiff	1	No
26	F-26	Pomeranian	6	Yes
27	F-27	Shih Tzu	5	Yes
28	F-28	Miniature Pinscher	7	No
29	F-29	Pomeranian	12	Yes
30	F-30	Pug	5	Yes
31	F-31	Labrador	6	No
32	F-32	Labrador	2	Yes
33	F-33	Pomeranian	12	Yes
34	F-34	Mongrel	6	Yes
35	F-35	German Shepherd	9	No
36	F-36	Shih Tzu	3	Yes
37	F-37	St. Bernard	12	Yes
38	F-38	Mongrel	30	No
39	F-39	Pomeranian	6	Yes
40	F-40	Labrador	1	No
41	F-41	Pomeranian	3	Yes
42	F-42	Golden retriever	12	No
43	F-43	Labrador	12	Yes
44	F-44	German Shepherd	5	Yes
45	F-45	Rottweiler	6	No
46	F-46	German Shepherd	12	Yes
47	F-47	German Shepherd	24	Yes
48	F-48	St. Bernard	2	Yes
49	F-49	German Shepherd	5	Yes
50	F-50	Daschund	6	Yes
51	F-51	Labrador	4	Yes
52	F-52	Daschund	1	No
53	F-53	Labrador	3	No

Table 1 (Cont.).

Sample no.	Sample ID	Breed	Age (months)	Vaccination status
54	F-54	Pomeranian	3	No
55	F-55	Rottweiler	5	Yes
56	F-56	Labrador	4	No
57	F-57	Pomeranian	9	No
58	F-58	Beagle	12	No
59	F-59	Pug	7	No
60	F-60	Labrador	1	Yes
61	F-61	German Shepherd	24	No
62	F-62	German Shepherd	12	Yes
63	F-63	Pomeranian	3	No
64	F-64	Labrador	48	No
65	F-65	Pomeranian	5	Yes
66	F-66	Mongrel	6	Yes
67	F-67	Beagle	8	Yes
68	F-68	Indian	4	Yes
69	F-69	Labrador	3	Yes
70	F-70	Pomeranian	6	No
71	F-71	Shih Tzu	31	Yes
72	F-72	Golden retreiver	7	Yes
73	F-73	Labrador	60	Yes
74	F-74	Mongrel	5	No
75	F-75	Pomeranian	8	No
76	F-76	Labrador	24	No
77	F-77	Shih Tzu	3	Yes
78	F-78	Shih Tzu	48	Yes
79	F-79	Doberman	6	Yes
80	F-80	Labrador	7	Yes
81	F-81	Rottweiler	60	Yes
82	F-82	Mongrel	15	Yes

Table 1 (Cont.).

Sample no.	Sample ID	Breed	Age (months)	Vaccination status
83	F-83	German Shepherd	3	No
84	F-84	Labrador	2	Yes
85	F-85	Mongrel	6	No
86	F-86	Labrador	96	Yes
87	F-87	Pug	2	No
88	F-88	Labrador	18	No
89	F-89	Labrador	1	No
90	F-90	Pomeranian	36	Yes
91	F-91	German Shepherd	2	No
92	F-92	Mongrel	4	No
93	F-93	Labrador	4	Yes
94	F-94	Mongrel	5	No
95	F-95	Shih Tzu	9	Yes
96	F-96	Mongrel	9	No
97	F-97	German Shepherd	7	Yes
98	F-98	Labrador	42	Yes
99	F-99	German Shepherd	7	Yes
100	F-100	Mongrel	4	No
101	F-101	Labrador	5	No
102	F-102	Pomeranian	6	Yes
103	F-103	German Shepherd	2	Yes
104	F-104	Pomeranian	12	Yes
105	F-105	Labrador	18	No
106	F-106	Mongrel	8	No
107	F-107	Shih Tzu	9	Yes
108	F-108	Pomeranian	20	Yes
109	F-109	German Shepherd	2	Yes
110	F-110	Beagle	8	Yes
111	F-111	Mongrel	5	Yes

Table 1 (Cont.).

Sample no.	Sample ID	Breed	Age (months)	Vaccination status
112	F-112	Labrador	6	No
113	F-113	Doberman	4	No
114	F-114	Pomeranian	3	Yes
115	F-115	German Shepherd	13	Yes
116	F-116	Mongrel	6	No
117	F-117	Labrador	3	Yes
118	F-118	Labrador	9	Yes
119	F-119	Pomeranian	12	No
120	F-120	German Shepherd	7	No
121	F-121	Doberman	1	Yes
122	F-122	Mongrel	24	No
123	F-123	Pomeranian	12	Yes
124	F-124	Beagle	6	No
125	F-125	Shih Tzu	9	Yes
126	F-126	Rottweiler	5	Yes
127	F-127	Labrador	4	No
128	F-128	German Shepherd	3.5	Yes
129	F-129	Pug	7	Yes
130	F-130	Pomeranian	8	No
131	F-131	Pomeranian	15	No
132	F-132	Mongrel	1.5	No
133	F-133	Doberman	4.5	Yes
134	F-134	German Shepherd	7	No
135	F-135	Beagle	1	Yes
136	F-136	Labrador	9	No
137	F-137	Shih Tzu	14	No
138	F-138	Pug	13	No
139	F-139	Rottweiler	13	No
140	F-140	Labrador	10.5	Yes

Table 1 (Cont.).

Sample no.	Sample ID	Breed	Age (months)	Vaccination status
141	F-141	Mongrel	8	No
142	F-142	Labrador	23	Yes
143	F-143	Doberman	3	Yes
144	F-144	German Shepherd	7	Yes
145	F-145	Pomeranian	4	No
146	F-146	Mongrel	2	No
147	F-147	Beagle	34	Yes
148	F-148	Labrador	8	Yes
149	F-149	Shih Tzu	5	Yes
150	F-150	Pomeranian	21	No

Processing of Samples

The clinical samples obtained from the suspected dogs were resuspendedin 3 mL of 0.1 M PBS (pH 7.4) containing antibiotics (100 IU/mL Benzyl Penicillin, 100 µg/mL streptomycin sulfate) and centrifuged at 10,000 rpm for 10 min at 4ºC. The supernatant was collected and filtered through a 0.22µm syringe filter and stored at -20ºC until further use.

EXTRACTION OF VIRAL NUCLEIC ACID

The following procedure was followed for RNA extraction.

- The 5 mL of fecal sample with PBS was centrifuged at 4000 rpm for 15 min at 4ºC to obtain pellet (Centrifuge 5810 R, Eppendorf).

- The pellet was collected into a 1.5mL microfuge tube along with 200 µLof supernatant, to which 750 µL of TRIZOL® reagent (Ref:15596018; Ambion®) was added.
- The mixture was vortexed for 1min and incubated at room temperature for 5 min.
- 250 µL of chloroform was added to the above mixture.
- The above mixture was hand mixed to avoid any cellular DNA contamination and incubated on ice for 10 min.
- The mixture was then centrifuged at 13,200 rpm for 15 min at 4 ºCusing a refrigerated centrifuge.
- The aqueous phase was collected carefully to another RNase free 1.5mL microfuge tube without disturbing the protein layer.
- An equal amount of ice-cold isopropyl alcohol was added to the collected aqueous phase.
- This mixture was incubated at -20 ºC for overnight.
- The following day, the mixture was centrifuged at 13,200 rpm for 15 min at 4 ºC.
- The supernatant was discarded leaving the small pellet (sometimes invisible).
- Then 1mL of 70% ethyl alcohol was added to the pellet and centrifuged at 13,200 rpm for 10 min at 4 ºC.
- The supernatant was discarded and the pellet was air- dried.
- The pellet was then resuspendedin 25 µL of nuclease-free water.

QUANTIFICATION AND QUALITY ASSESSMENT OF RNA

- RNA was quantified by NanoDrop TM Lite Spectrophotometer (Thermo Fisher Scientific).
- For nucleic acid quantification, the Beer-Lambert equation was modified to use a conversion factor with units of ng-cm/µl. the modified equation for nucleic acid calculations is the following:
- C = (A*CF)/l Where,
- C = the nucleic acid concentration in ng/µl A = the sample absorbance
- CF = the conversion factor in ng-cm/µl = the pathlength in cm
- The U.V absorbance was checked at 260 and 280 nm wavelength for determination of sample concentration and purity. Purity of RNA was judged based on the O.D. ratio at 260:280. The RNA having a ratio of 2.0 was considered to be of good purity.

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

RT-PCR was done in two steps. First cDNA synthesis was carried out and the synthesized cDNA was used for PCR.

Reverse Transcription

The reaction was carried out using the PrimeScript™ 1st strand cDNA Synthesis kit (Cat No.6110A; TaKaRa). A 20 µL of RNA of each sample was converted to 40 µL of cDNA, in the following steps.

- The reagents were allowed to thaw completely. Then were mixed gently and spun briefly. The master mix was prepared by scaling up based on the volumes listed below to the desired number ofreactions.
- The following mixture was prepared in a microfuge tube.

Reagent	Volume
Random hexamers (0.1µM)	2 µL
dNTP Mixture (10mM each)	2 µL
Template RNA (<5µg)	20 µL
RNase free dH2O	6 µL
Total	30 µL

- This Template RNA-Primer Mixture was incubated for 5 min at 65 °C and then snap cooled on ice.
- The following reaction mixture was prepared.

Reagent	Volume
Template RNA andPrimer Mixture	30 µL
5X PrimeScripit Buffer	8 µL
RNase Inhibitor (20 units)	1 µL
PrimeScriptRTase (100-200 units)	1 µL
Total	40 µL

- This reaction mixture was added into the processed template RNA-Primer

Mixture by mixing gently and then incubated at the following conditions in a thermal cycler (Prima- Duo,HiMedia).

25 ºC	10 min
42 ºC	60 min
70 ºC	10 min (forenzyme inactivation)
4 ºC	∞

PCR

PCR was carried out using Emerald Amp® GT PCR Master Mix (RR310A; TaKaRa) with a set of forward and reverse primers of CAstV- ORF-1b and CDV-partial H which amplifies 293 bp of ORF-1b gene and 449 bp of partial H gene respectively (Table 3.2).The primers were obtained in lyophilised 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 standardised for the primer set of ORF-1b as per Martella*et al.*(2011) and the CDV-partial H gene was standardised as reported by Ashmi*et al.* (2017).

- 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.
- Using the template cDNA, PCR mixture was prepared as follows.

Reagent	**Volume**
Emerald Amp® GT PCR Master Mix (2X Premix)	12.5 µL
Forward Primer (0.2 µM)	1 µL
Reverse Primer (0.2 µM)	1 µL

Template cDNA (<500 ng)	2 µL
dH_2O(Sterile distilled water)	8.5 µL
Total	25 µL

- The above-mentioned contents were transferred to a 0.2 ml tube on ice. The PCR mixture was mixed thoroughly by using a pipette and was spunbriefly.
- The tube was placed in a thermal cycler and the following cycling conditions for the primer set were followed.
- Then 1% gel electrophoresis was carried out and the PCR product was confirmed by comparison of migration distances with 100bpDNA ladder (Cat.No.3422A; TaKaRa),along with positive template control and No Template Control (NTC); using gel documentation system.

S.No	**Steps**	**CDV**	**CAstV**
1	Initial denaturation	95ºC for 5 min	95ºC for 5 min
2	Denaturation	95ºC for 30 sec	95ºC for 30 sec
3	Annealing	54ºC for 30 sec	56ºC for 30 sec
4	Initial extension	72ºC for 30 sec	72ºC for 30 sec
		Steps 2-4 35 cycles	
5	Final extension	72ºC for 10 min	72ºC for 10 min
6	Hold	4ºC	∞

Table: 3.2 Conventional primer pairs used for amplification of partial *H gene* of CDV and *ORF-1b gene* of CAstV.

S. No.	Forward and reverse primers	Primer Sequence (5'-3') direction	Gene amplified	Position of the genome	Annealing temperature and product size
1	CDV partial H (F) CDV partial H (R)	TACTGAATGGAGAC GGTATGGAT TCGATAGAATTGGTG ACATCACA	partial H-gene	8379-8401 8827-8805	53°C 449 bp
2	CAstV ORF-1b (F) CAstV ORF-1b (R)	GTACTATACCRTCTG ATTTAATT AGACCAARGTGTCAT AGTTCAG	ORF-1b	3469-3491 3741-3762	54°C 293 bp

AGAROSE GEL ELECTROPHORESIS

- One g of Agarose (MB002-500G; HiMedia) powder was resuspendedin 100 mL TAE buffer and heated in a microwave oven until the agarose meltscompletely.
- After letting it cool down to 50^{o}C, Ethidium Bromide (E8751-10G; Sigma-Aldrich) was added to a final concentration of 0.5 µg/mL. The content was swirled gently to mix the reagents.
- The molten agarose was poured into a casting tray and after positioning the required comb; it was allowed to solidify for at least 20 min.
- The comb was removed gently and the gel tray was positioned in the electrophoresis tank and the buffer was added so that the gel was just submerged within the buffer.

- Five µL of each PCR amplicon with 1 µL of Gel Loading Dye, Purple (6X) (Cat.No.B7024S; New England BioLabs) and 100bp DNA ladder (Cat. No. 3422A; TaKaRa) were loaded inwells.
- Electrophoresis was carried out at 80 volts for 1hr.
- The gel was transferred to the gel documentation system (Gene flash, Syngenebioimaging) having UV trans-illuminator and observed under medium wavelength for the desired bands.

POSITIVE CONTROL

- Commercially available live attenuated vaccine for CDV; Canishot K5 (Rockborn strain) was used as a positive control. The vaccine RNA of CDV was extracted using the above-mentioned extraction procedure.
- Previously isolated CAstV sample in our college was used as a positive control.

PROPAGATION OF VIRUS FROMFECAL SAMPLES IN CELL CULTURE

The clinical samples which tested as positive for CDV and CAstV in the PCR were processed for virus isolation in B95a cell line and MDCK cell lines respectively.

Infecting the B95a and MDCK cells

A T_{25}flask with 70% monolayer was processed for infection. The spent growth medium was decanted and the monolayer was washed twice with 1X PBS. To it, 1 mL of the filtered fecal sample was added to the flask and incubated for 1hr at 37ºC with 5% CO_2 while gentle shaking or every 15 min to ensure uniform adsorption. After 1 hr of adsorption, the inoculum was decanted and 5-7 mL maintenance medium containing 1% FBS was added. The flask was incubated in an incubator at 37ºC with 5% CO_2 and observed at 24 hrs interval for CPE. CPE was noted within 2-5 days as rounding and detachment of cell bunches in the infected flask, in comparison to a healthy monolayer in the control flask containing only maintenance medium. Flasks that show little or no CPE were continued to culture up to 7 days, by replacing half the amount of medium with fresh maintenance medium at 1-day interval until any development of CPE.

After 90% detachment of monolayer in infected flasks, cells were lysed by two cycles of freeze/thaw. Then the culture fluid was centrifuged at 3000 rpm for 10 min and the collected supernatant was stored at 4ºC. This culture fluid was used further passagedin B95acells.

FREEZE THAWING OF CELL CULTURE FLUID

For a 90% detachment of monolayer in infected flasks, the cells were freeze/thawed for two times. Freeze-thaw involved rapid freezing at -80ºC for 20 min and rapid thawing to 37 ºC in a water bath.

SEQUENCING

PCR for Sequencing

PCR was carried out using Emerald Amp® GT PCR Master Mix (RR310A; TaKaRa) with a set of respective forward and reverse primers which amplifies 449 bpof partial H gene of CDV and 297bp of ORF-1b gene of CAstV.

Purification of the PCR product for sequencing

- To 1 volume of the PCR product, 5 volumes of PB buffer (supplied with the QIAquickkit)was added in micro-centrifuge tubes and mixed by inverting the tubes severaltimes.
- Sample was then transferred to the QIAquick® spin column and centrifuged for1 min at 13,200 rpm and the flow-through wasdiscarded.
- 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 13,200rpm.
- QIAquick® spin columns were placed in a fresh 1.5 mLmicro-centrifugetube.
- The PCR product was eluted by adding 30µLof nuclease-free water/EB buffer to the center of the membrane, columns were kept standstill for 5 min and then centrifuged for 1 min at 13,200 rpm. Eluted product was quantified by NanoDrop TM Lite Spectrophotometer (Thermo FisherScientific).

Nucleotide Sequencing of Partial H-gene of CDV and ORF-1b gene of CAstV

The DNA sequencing was done at sequencing facilities Bioserve, Hyderabad.

WHOLE GENOME SEQUENCING

The cell culture positive isolate was given for whole genome sequencing at MedGenome Labs Ltd. Bengaluru, Karnataka 560099.

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 CAstV and 14 Gb data was generated with 95 million reads for CDV. 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

The sequences of CDV and CAstVwhole genome, CDV-partial H gene and CAstV ORF-1b gene isolates obtained after sequencing were compared against other

available sequences in GenBank using NCBI BLAST (www.ncbi.nlm.nih/gov/blast).

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 no. of replicates as 1000. The clades were divided according to geographical distribution or as per relativeness in to different lineages/groups.

CHAPTER IV

RESULTS

In the present investigation, attempts were made to study the prevalence of, and isolate CDV and CAstV from the clinical cases of canine gastroenteritis along with the whole genome characterization of CDV and CAstV. The phylogenetic analysis of the isolates was done by comparing the sequences with available NCBI database sequences. The detailed results are presented below.

MOLECULAR DETECTION OF CDV AND CAstV

A total of 150 fecal samples/rectal swabs obtained from suspected dogs were processed and RNA extraction was done. Commercially available vaccine (Canishot K5) served as a positive control for CDV.

A total of 150 cDNA samples were screened by PCR using primers targeting partial-H gene for CDV, and primers targeting ORF-1b gene for CAstV. A total of five (3.3%), and six (4%) samples out of 150 fecal samples tested were positive for CDV (Table. 4.1),and CAstV, respectively (Table. 4.2).Agarose gel electrophoresis pattern of CDV detection(Fig. 4.1) and CAstVdetection(Fig. 4.2) is shown below.

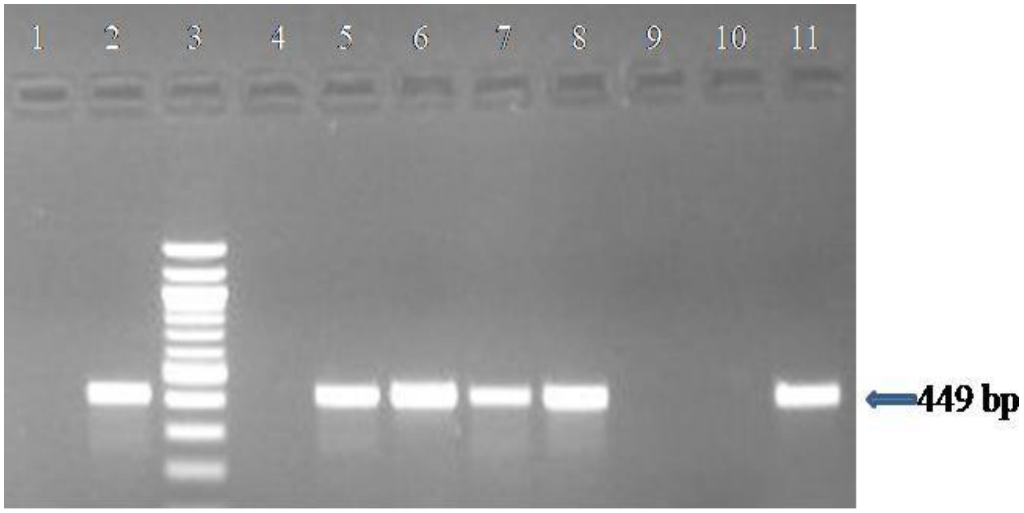


Fig. 4.1 PCR amplicon of CDV-H gene resolved in 1% agarose gel

The amplicon size of CDV H-gene is 449bp.

Lane 1: Negative control
Lane 2: Positive control
Lane 3: Marker (100bp)
Lane 4: F-26
Lane 5: F-11
Lane 6: F-13
Lane 7: F-92
Lane 8: F-105
Lane 9: F-122
Lane 10: F-136
Lane 11: F-141

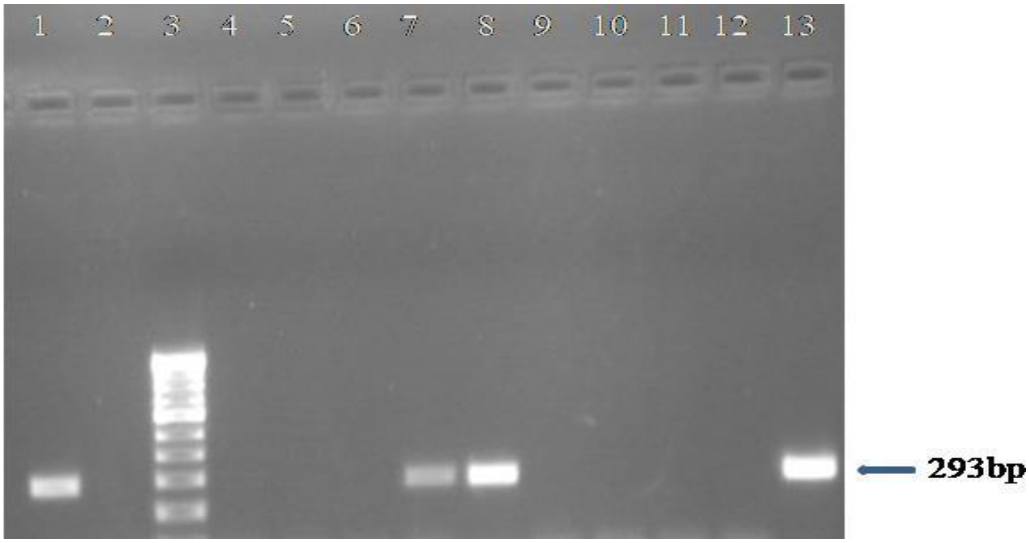


Fig.4.2 Representative image of PCR amplicon of CAstV ORF-1b gene resolved in 1% agarose gel

The amplicon size of CAstV ORF-1b gene is 293bp.

Lane 1: Positive control
Lane 2: Negative control
Lane 3: Marker (100bp)
Lane 4: F-31
Lane 5: F-64
Lane 6: F-73
Lane 7: F-48
Lane 8: F-93
Lane 9: F-109
Lane 10: F-128
Lane 11: F-130
Lane 12: F-161
Lane 13: F-101

ISOLATION OF CDV AND CAstV

Isolation of CDV in B95a Cell Line

Two out of five cultured fecal samples which were tested positive for CDV in the PCR showed a characteristic CPE of CDV in the first passage in B95a cell line (Table. 4.1).The uninfected monolayer did not show any change until 10 days of incubation except for a few detached cells due to overgrowth of cells (Fig. 4.3A).The CPE was observed in 5 days post-infection with rounding of cells, ballooning and detachment of cell bunches in infected flasks (Fig. 4.3B),in comparison to healthy monolayer in the control flask with maintenance medium.

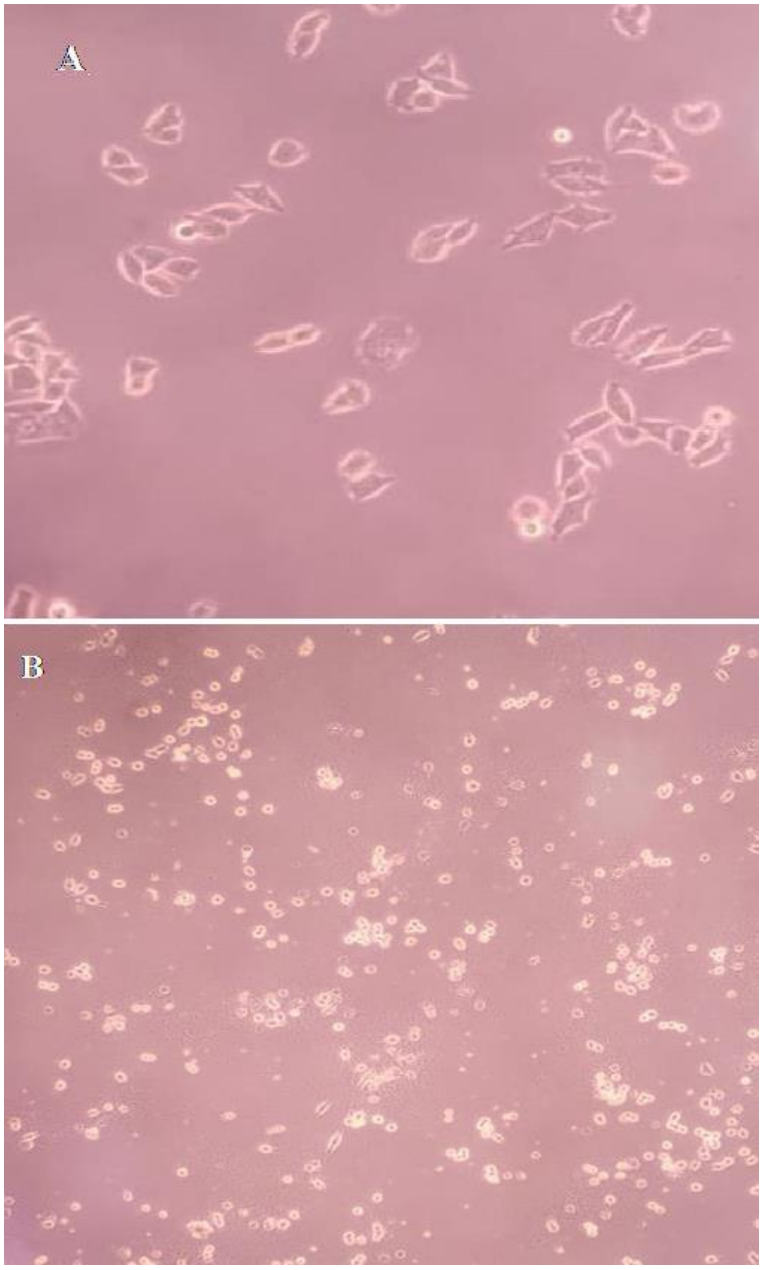


Fig. 4.3 Isolation of CDV in B-95a cell line

(A) Uninfected B95a monolayer cell line and (B) infected B95a monolayer cell line showing CPE (100X) after 7 days.

Isolation of CAstV in MDCK Cell Line

All the six cultured fecal samples which were tested positive for CAstVin the PCR showed a characteristic CPE of CAstVin MDCK cell line (Table. 4.2).The uninfected monolayer did not show any change until 10 days of incubation except for a few detached cells due to overcrowding of cells (Fig. 4.4A).The initiation of CPE was observed as early as 72 hrs post-infection, with initial rounding and detachment of cell bunches in infected flasks, in comparison to healthy monolayer in the control flask with maintenance medium. Finally, complete detachment of the cells from the surface occurred and the detached cells appeared as bunches of grapes in infected flasks (Fig. 4.4B).

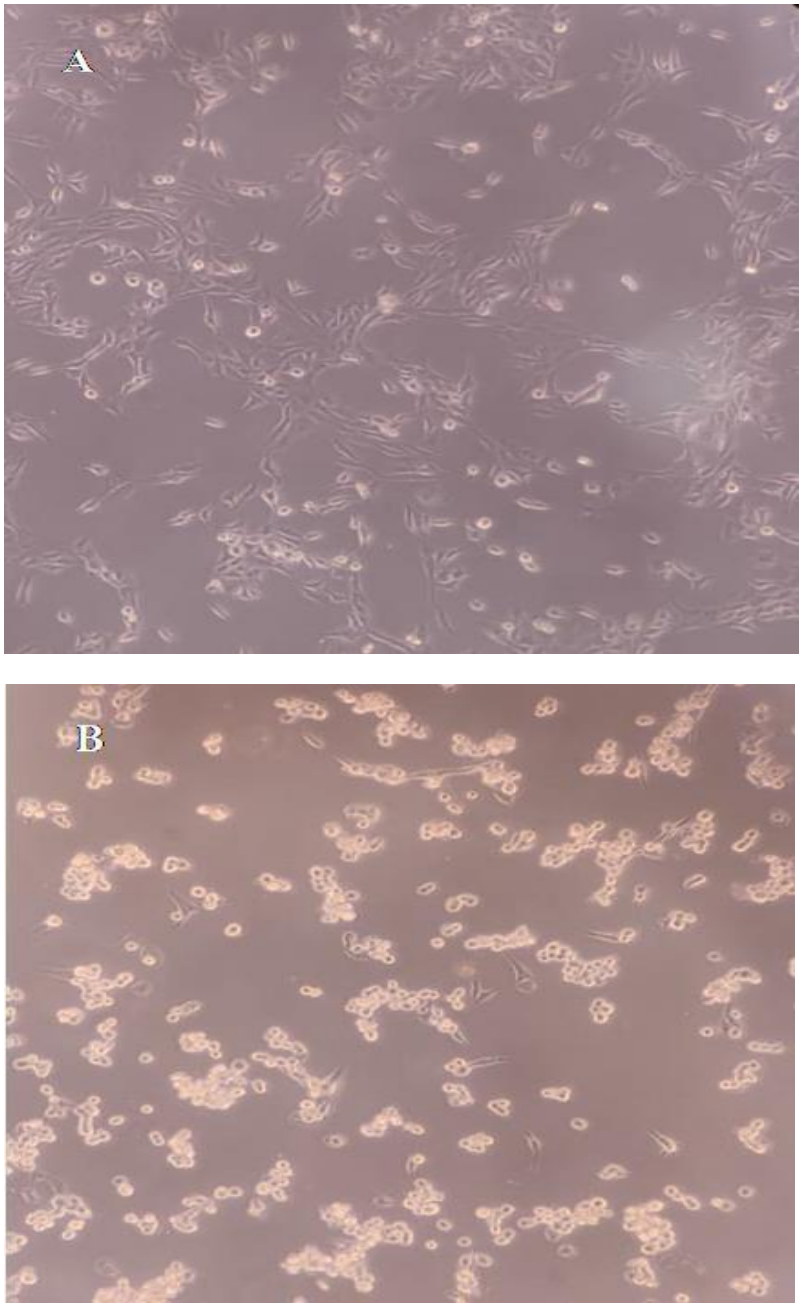


Fig. 4.4Isolation of CAstV in MDCK cell line

(A) Uninfected MDCK monolayer cell line and (B) infected MDCK monolayer cell line showing CPE (100X) after 5 days.

Table 4.1 Results of CDV detection and isolation

S.No.	Sample ID.	Breed	Age(months)	Clinical samples (+) in PCR	CDV (+) samples shown CPE in cell culture
1.	F-11	German Shepherd	4	Yes	No
2.	F-13	German Shepherd	6	Yes	Yes
3.	F-92	Mongrel	4	Yes	Yes
4.	F-105	Labrador	18	Yes	No
5.	F-141	Mongrel	8	Yes	No

Table 4.2 Results of CAstV detection and isolation

S.No.	Sample ID.	Breed	Age (months)	Clinical samples(+) in PCR	CAstV (+) samples shown CPE in cell culture
1.	F-32	Labrador	2	Yes	Yes
2.	F-48	St. Bernard	2	Yes	Yes
3.	F-77	Shih Tzu	3	Yes	Yes
4.	F-93	Mongrel	4	Yes	Yes
5.	F-101	Labrador	5	Yes	Yes
6.	F-132	Mongrel	1.5	Yes	Yes

CONFIRMATION OF CDV ISOLATES BY PCR

Partial H gene of 449 bp was amplified in one CDV isolate. The size of the amplified product was analyzed by AGE by employing standard DNA molecular size markers. No amplification of similar size was observed in negative control indicating that the amplified products were specific for partial H gene of CDV (Fig. 4.5).

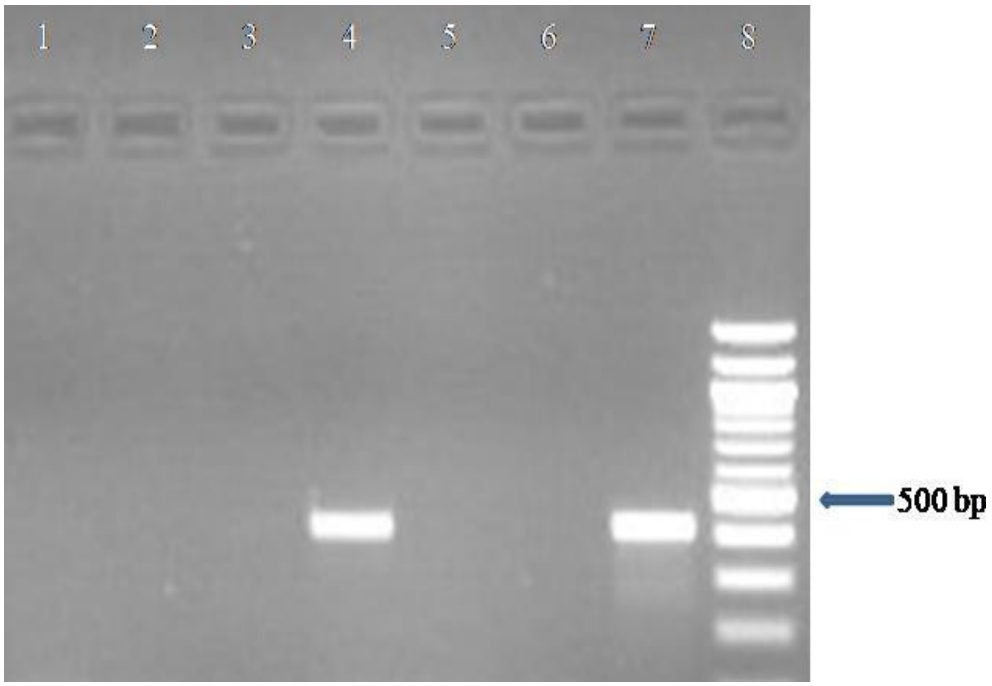


Fig. 4.5 PCR amplicon of CDV-H gene resolved in 1% agarose gel

The amplicon size of CDV is 449bp.

Lane 1: F-11
Lane 2: F-13
Lane 3: F-92
Lane 4: F-105
Lane 5: F-141
Lane 6: Negative control
Lane 7: Positive control
Lane 8: Marker (100bp)

CONFIRMATION OF CAstV ISOLATES BY PCR

ORF-1b gene of 293bp was amplified in one isolate. The size of the amplified product was analyzed by AGE by employing standard DNA molecular size markers. No amplification of similar size was observed in negative control indicating that the amplified products were specific for ORF-1b gene ofCAstV(Fig. 4.6).

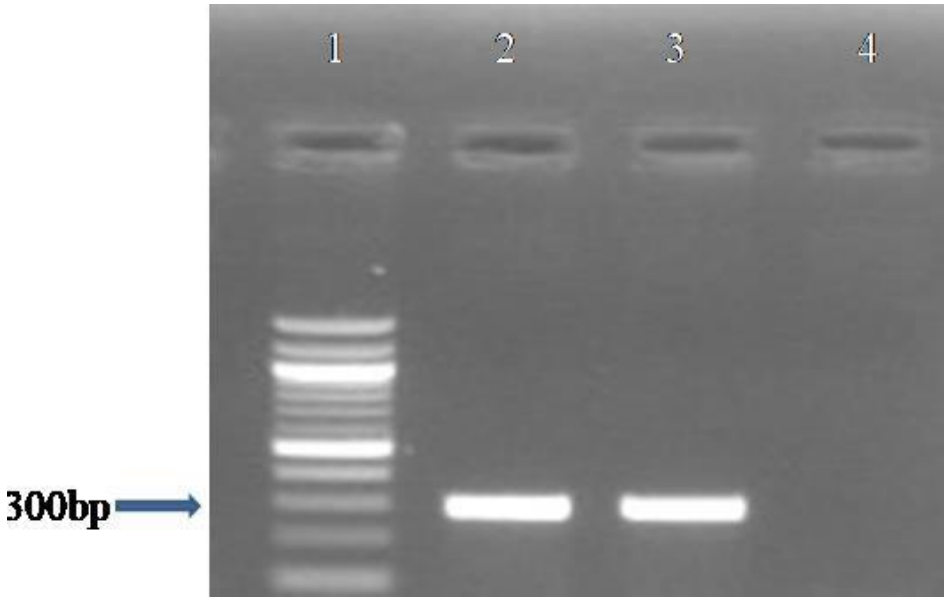


Fig. 4.6**PCR amplicon of CAstV ORF-1b gene resolved in 1% agarose gel**
The amplicon size of CAstV is 293bp
Lane 1: Marker (100bp) Lane 3: Positive control
Lane 2: F-48 Lane 4: Negative control

PARTIAL GENE SEQUENCINGOF CDV AND CAstV

Sequencing

PCR product of partial H gene of size 449 bp (1 Nos) and ORF-1b gene of size 295bp (4 Nos) was sequenced at sequencing facilities, Bioserve Biotechnologies India Pvt Ltd, Hyderabad using the primers used for PCR amplification. The generated forward sequence chromatograms were initially edited with Chromas V 2.0 software and low quality sequences were trimmed from raw sequence data file, a representative chromatogram image showing nucleotide peaks were represented in Fig. 4.7.The sequences were submitted to GenBank and the NCBI accession numbers are as follows:

MT955600 PVNRTVU/2020/CAstV 01- F:48,

MT955601 PVNRTVU/2020/CAstV 02- F:77,

MT955603 PVNRTVU/2020/CAstV 03- F:93,

MT955602 PVNRTVU/2020/CAstV 04- F:101,

MW014320 PVNRTVU/2020/CDV partial-H gene-F:105.

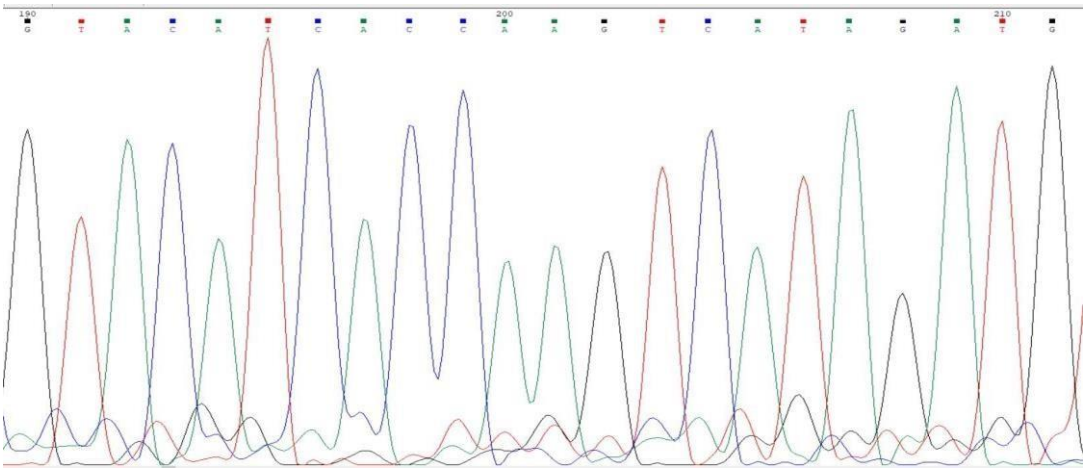


Fig. 4.7 Chromatogram as visualized in chromas V 2.0.
The good quality sequences were taken, which are distinguishable clearly with high peaks to study the evolutionary relationship of isolates selected for sequencing from the current study with other isolates from different geographical regions of the world.

Sequence Analysis

The sequences of partial-H (CDV) and ORF-1b (CAstV) obtained after sequencing were compared against other available sequences from GenBank using NCBI BLAST. After retrieving the sequences from the chromatogram, the sequences were aligned against the other isolates from the NCBI database using MUSCLE from MEGA7. The percentage nucleotide sequence identity ofcurrent CDV isolates with other isolates was compared using NCBI BLAST. Further, theamino acid sequences of the isolates were aligned with other available sequences tounderstand the variations in the protein sequence.

Nucleotide sequencing of partial ORF-1b gene of CAstV isolates PVNRTVU/2020/CAstV 01-04 were compared against reference strains which revealed that there is 97-99% nucleotide and 99-100% amino acid identity with each other and 91-100% nucleotide identity and 97-99% amino acid identity with other sequences in GenBank database.

Nucleotide sequencing of partial-H gene of CDV isolate with reference to vaccine strains and other sequences of CDV isolates from GenBank showed 90 -95% nucleotide identity and 85-91% amino acid identity.

Multiple Sequence Alignment of CDV-H gene

Using MUSCLE in MEGA 7.0, the multiple sequence alignment was generated by comparing nucleotide sequence of CDV isolates from current study against other sequences of CDV obtained from GenBank (Fig. 4.8) which revealed that there is presence of Nde-I restriction site at position of 1571-1576 that is typical for wild strains of CDV.

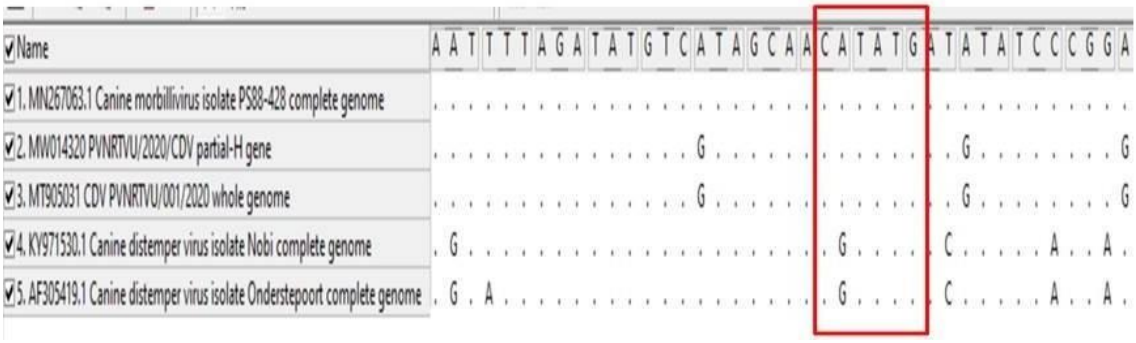


Fig. 4.8Multiple sequence alignment of partial *H* gene of CDV isolates with reference to India strains and vaccine strains.
The partial *H* gene show *NdeI*restriction site (CATATG) at the position of 1571-1576 for wildtype strains whereas the site was absent due to substitution of A to G (CGTATG) in vaccine strains.

WHOLE GENOME SEQUENCING OF CDV AND CAstV

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 CAstV and 14 Gb data was generated with 95 million reads for CDV. 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 and the accession numbers are: MT905031-CDV PVNRTVU/001/2020 and MT894143-PVNRTVU/CAstV/001/2020 forCAstV.

PHYLOGENETIC ANALYSIS OF CDV

Whole genome sequence of CDVisolated in the current study (CDVPVNRTVU/001/2020) was compared with the CDV sequences belonging to different geographical regions obtained from the GenBank like CDV VR-1587 (GenBank Acc. No.JN896987.1) CDV Synder Hill (GenBank Acc. No.GU138403.1) ondersteport vaccine (GenBank Acc. No.AF305419.1) Bucharest vaccine (GenBank Acc. No.KY971529.1) CDV 98-2646 (GenBank Acc. No.AY542312.2) CDV 98-2654 (GenBank Acc. No. AY466011.2)CDV 98-2645 (GenBank Acc.

No.AY445077.2) CDV 00-2601 (GenBank Acc. No.AY443350.1) CDV 01-2689 (GenBank Acc. No.AY649446.1) CDV 164071 (GenBank Acc. No.EU716337.1)CDV A75/17 (GenBank Acc. No.AF164967.1) CDV5804P (GenBank Acc. No.AY386316.1)CDV 5804 (GenBank Acc. No.AY386315.1)CDV HLJ1-06 (GenBank Acc. No.JX681125.1) CDV MKY-KM08 (GenBank Acc. No.HM852904.1)CDV C YN07-hV (GenBank Acc. No.AB687721.2) CDV C YN07-dV (GenBank Acc. No.AB687720.2) CDV WT01SA (GenBank Acc. No.KY971528.1) CDV WT01SA(GenBank Acc. No. KY971532.1) CDV 50Con (GenBank Acc. No.AB476402.1)CDV 011C (GenBank Acc. No.AB476401.1) CDV M25CR (GenBank Acc. No.AB475097.1)CDV 007Lm-1vp (GenBank Acc. No.AB462810.1) CDV007Lm (GenBank Acc. No. AB474397.1). The results were shown in Fig.4.9.

Based on the clustering of isolates from different geographical locations, they were divided into different lineages like Asia-1, Asia-2, America-1, America-2, Africa and vaccine. The whole genome isolate of the current study i.e. CDV/PVNRTVU/001/2020 clustered into Asia-1 lineage.

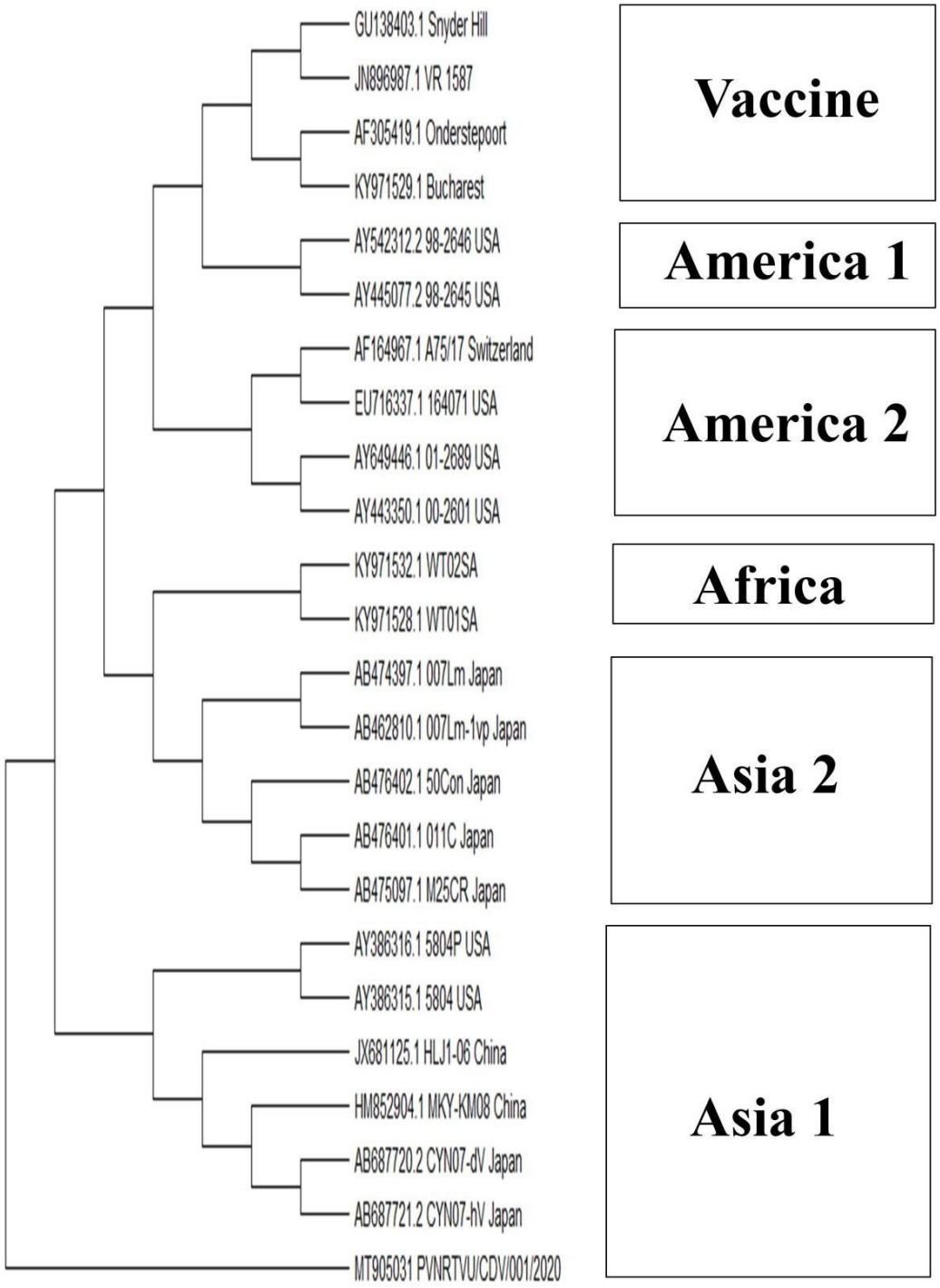


Fig. 4.9 Phylogenetic analysis of CDV whole genome

The phylogenetic tree was constructed by using the maximum likelihood method on Tamura Nei model. The tree shows the relationship between the current isolate MT905031 PVNRTVU/CDV/001/2020 and other isolates from different countries. The current isolate clustered into the ASIA-1 lineage and all the vaccines clustered into separate lineage distinct from the current isolate.

In addition to the phylogenetic analysis of the whole genome, phylogenetic trees of individual gene were also constructed to find any variation between the individual genes by using the same isolates used for whole genome sequencing.The N-gene (Fig. 4.10), P-gene (Fig. 4.11), M-gene (Fig. 4.12), F-gene (Fig. 4.13), H-gene (Fig. 4.14), L-gene (Fig. 4.15) phylogenetic trees are represented below. The current isolate clustered into Asia-1, Asia-2, and America lineages based on the individual gene, which are distinct from the vaccine lineage. For, N-gene, the phylogenetic tree was constructed and divided into different lineages. The isolate of the current study clustered into Asia-1 lineage shown in Fig. 4.10. For P-gene, the phylogenetic tree was constructed and divided into different lineages. The isolate of the current study clustered into Asia-2 lineage shown in Fig. 4.11. For M-gene, the phylogenetic tree was constructed and divided into different lineages. The isolate of the current study clustered into Asia-1 lineage shown in Fig. 4.12. For F-gene, the phylogenetic tree was constructed and divided into different lineages. The isolate of the current study clustered into Asia-1 lineage shown in Fig. 4.13. For H-gene, the phylogenetic tree was constructed and divided into different lineages. The isolate of the current study clustered into America-1 lineage shown in Fig. 4.14. For L-gene, the phylogenetic tree was constructed and divided into different lineages. The isolate of the current study clustered into Asia-1 lineage shown in Fig. 4.15.

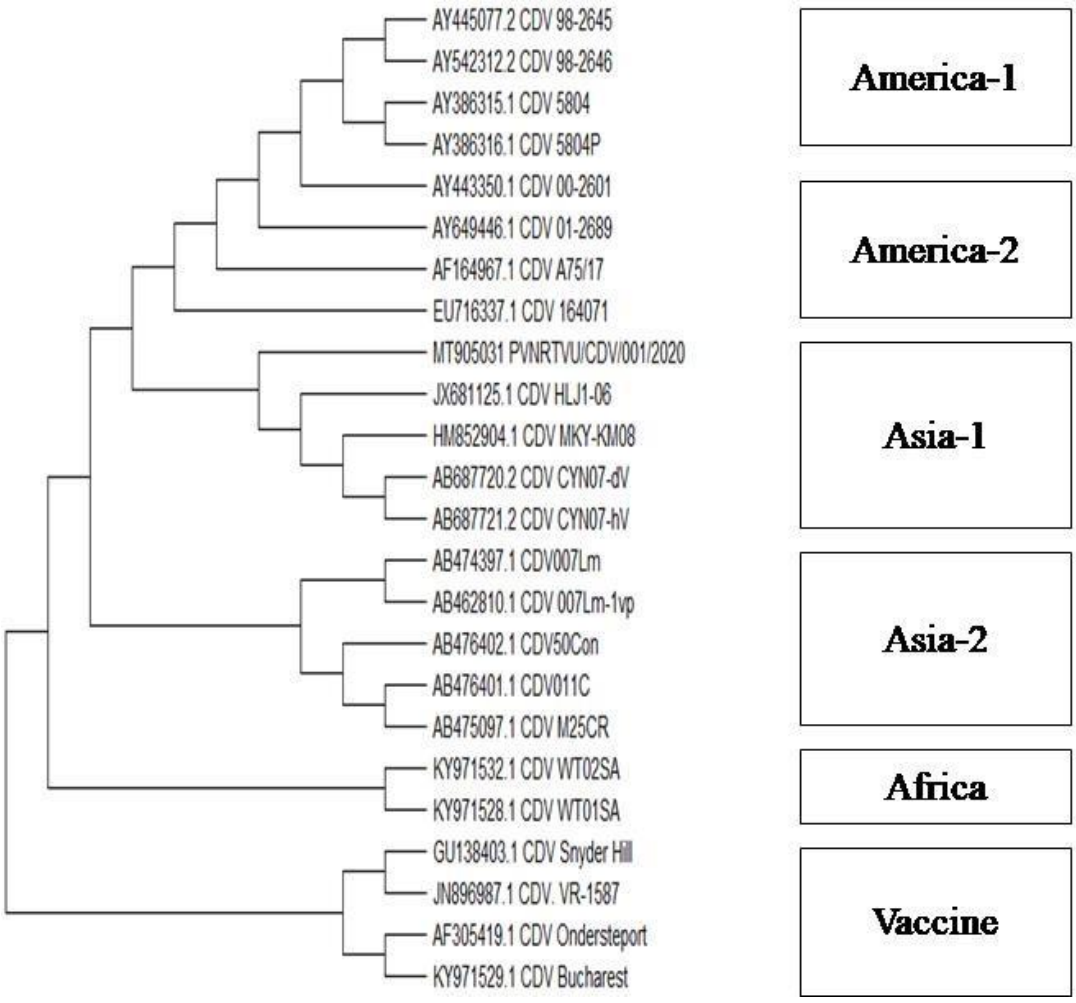


Fig. 4.10 Phylogenetic analysis of N-gene of CDV

The phylogenetic tree was constructed by using the maximum likelihood method on Tamura Nei model. The tree shows the relationship between the current isolate MT905031 PVNRTVU/CDV/001/2020 and other isolates from different countries. The current isolate clustered into the ASIA-1 lineage and all the vaccines clustered into separate lineage distinct from the current isolate.

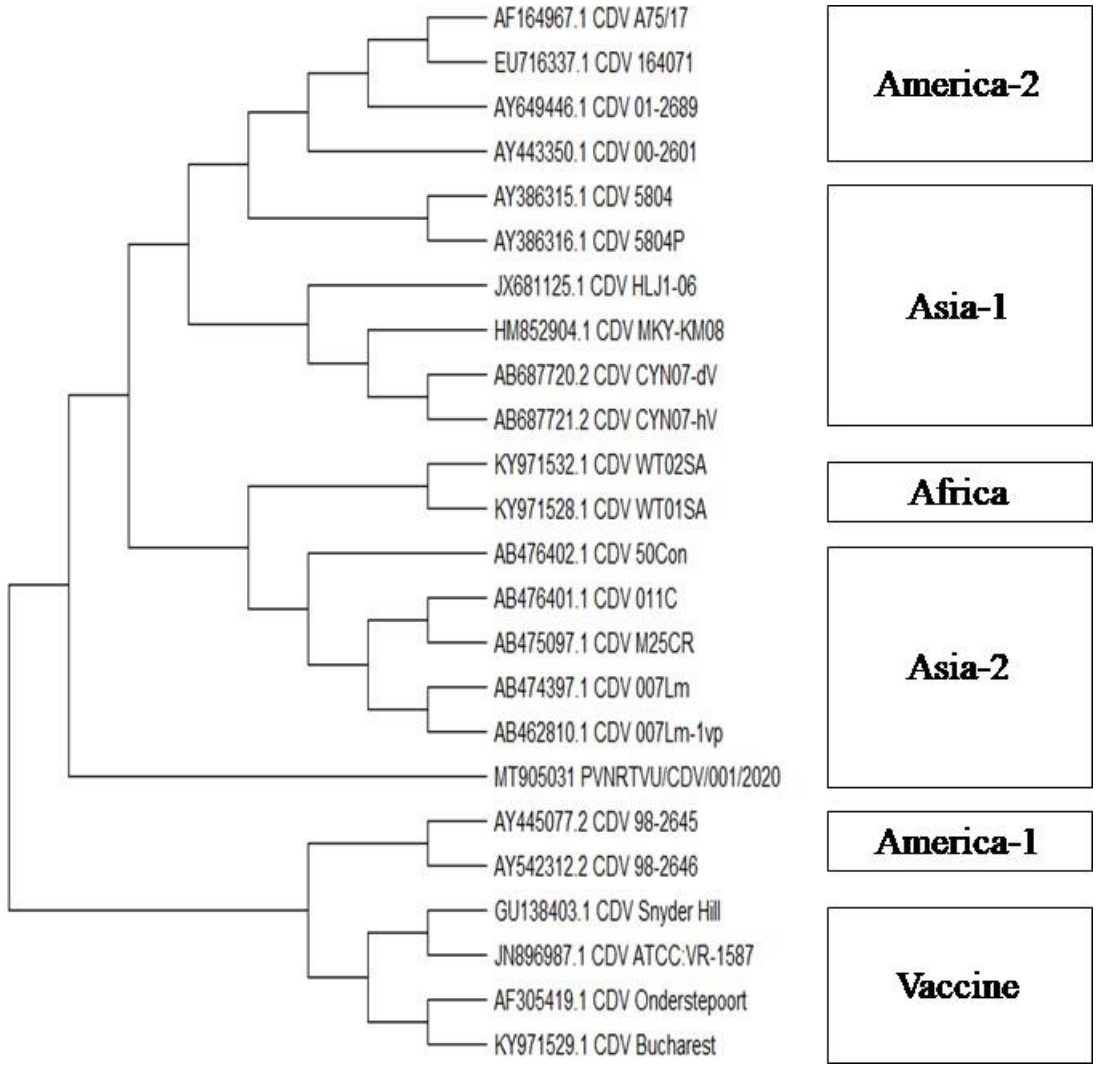


Fig. 4.11 Phylogenetic analysis of P-gene of CDV

The phylogenetic tree was constructed by using the maximum likelihood method on Tamura Nei model. The tree shows the relationship between the current isolate MT905031 PVNRTVU/CDV/001/2020 and other isolates from different countries. The current isolate clustered into the ASIA-2 lineage and all the vaccines clustered into separate lineage distinct from the current isolate.

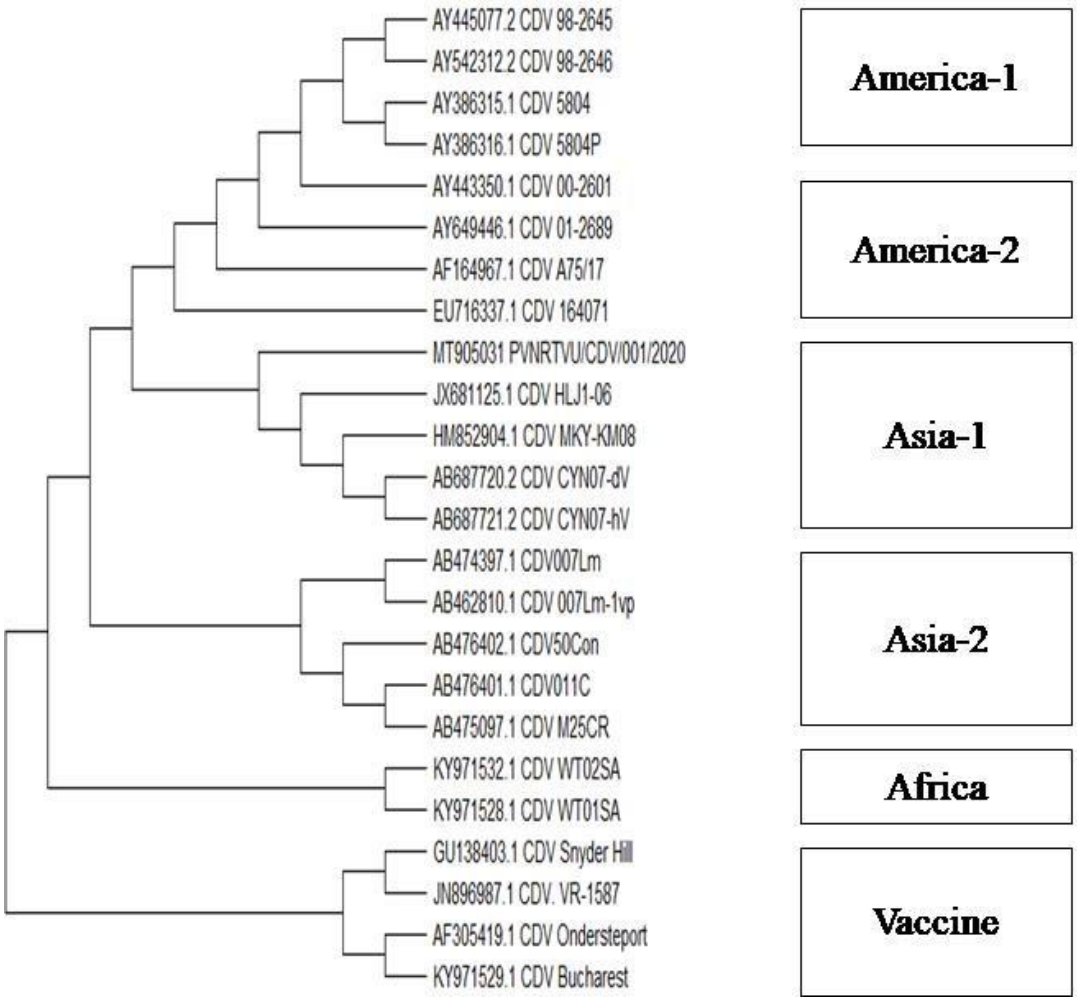


Fig. 4.12 Phylogenetic analysis of M-gene of CDV

The phylogenetic tree was constructed by using the maximum likelihood method on Tamura Nei model. The tree shows the relationship between the current isolate MT905031 PVNRTVU/CDV/001/2020 and other isolates from different countries. The current isolate clustered into the ASIA-1 lineage and all the vaccines clustered into separate lineage distinct from the current isolate.

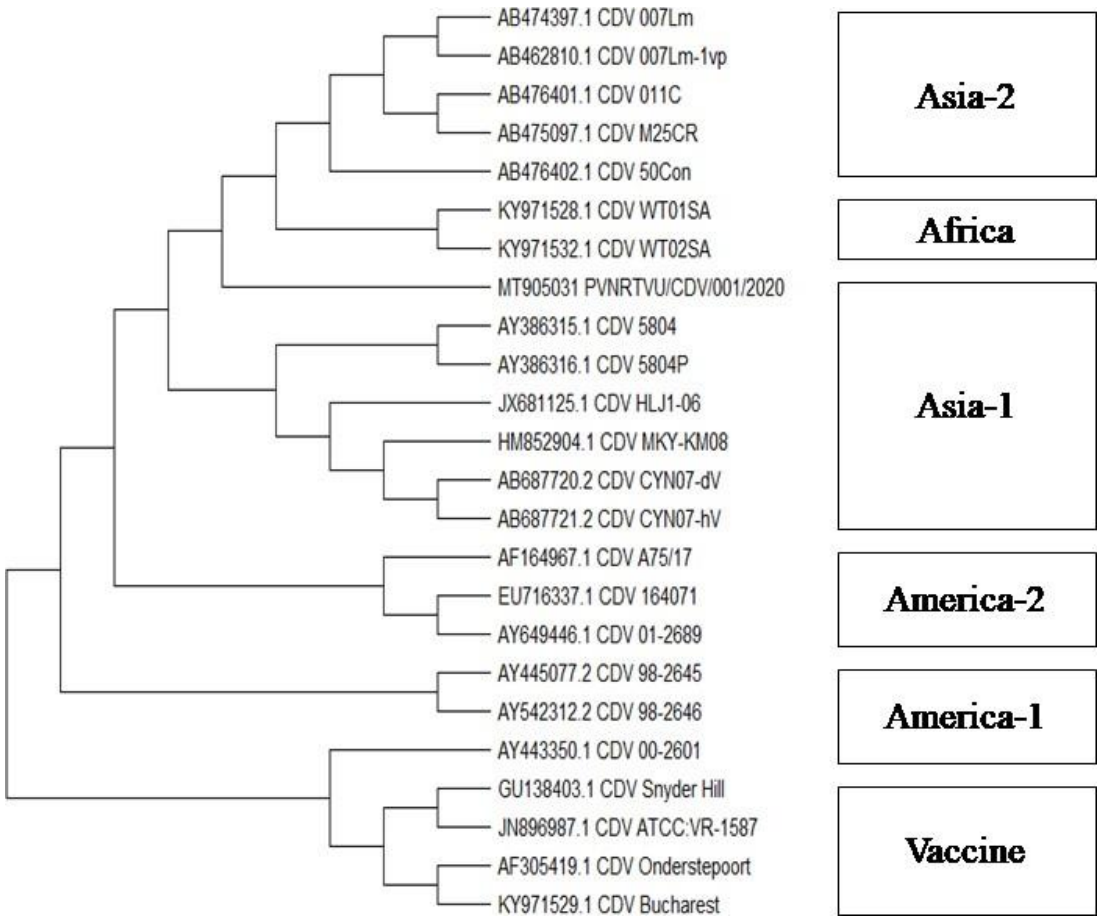


Fig. 4.13 Phylogenetic analysis of F-gene of CDV

The phylogenetic tree was constructed by using the maximum likelihood method on Tamura Nei model. The tree shows the relationship between the current isolate MT905031 PVNRTVU/CDV/001/2020 and other isolates from different countries. The current isolate clustered into the ASIA-1 lineage and all the vaccines clustered into separate lineage distinct from the current isolate.

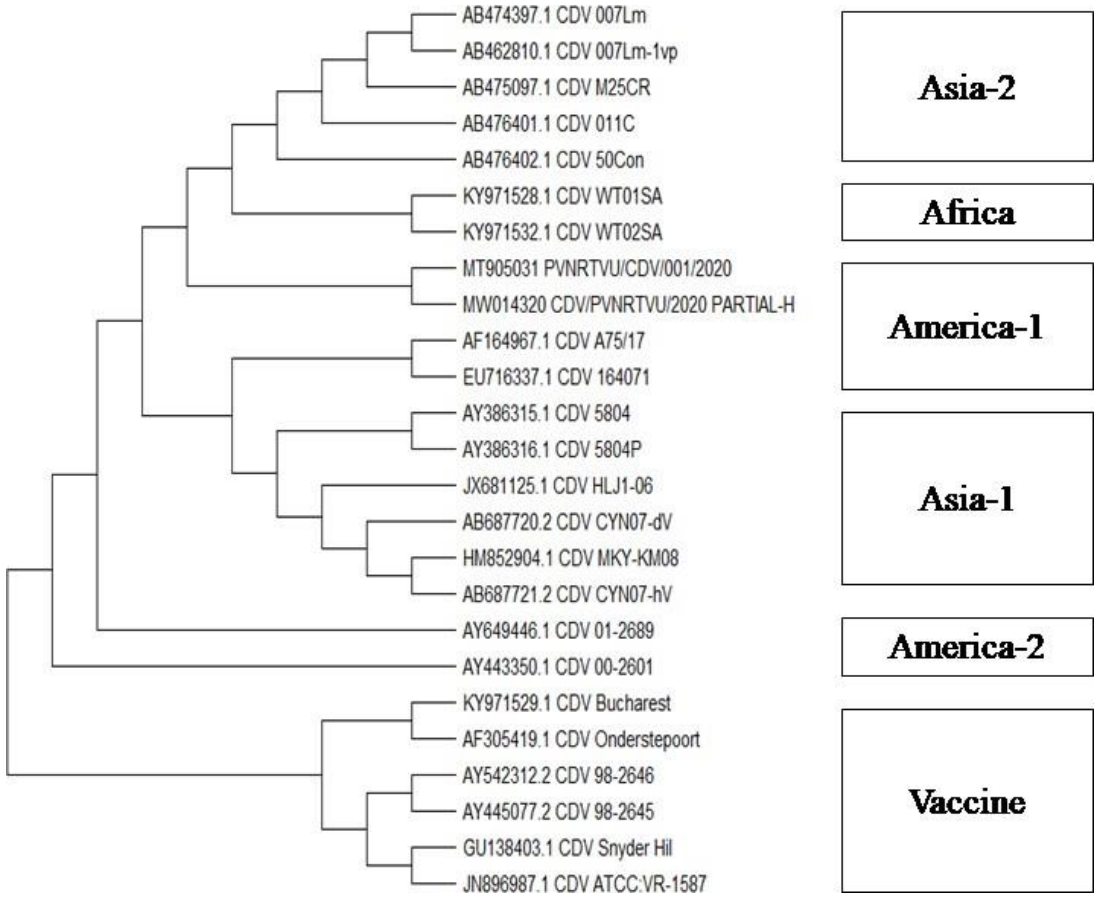


Fig. 4.14 Phylogenetic analysis of H-gene of CDV

The phylogenetic tree was constructed by using the maximum likelihood method on Tamura Nei model. The tree shows the relationship between the current isolate MT905031 PVNRTVU/CDV/001/2020 and other isolates from different countries. The whole genome isolate and the partial H-gene isolate clustered into America-1 lineage which is distinct from the vaccine strains.

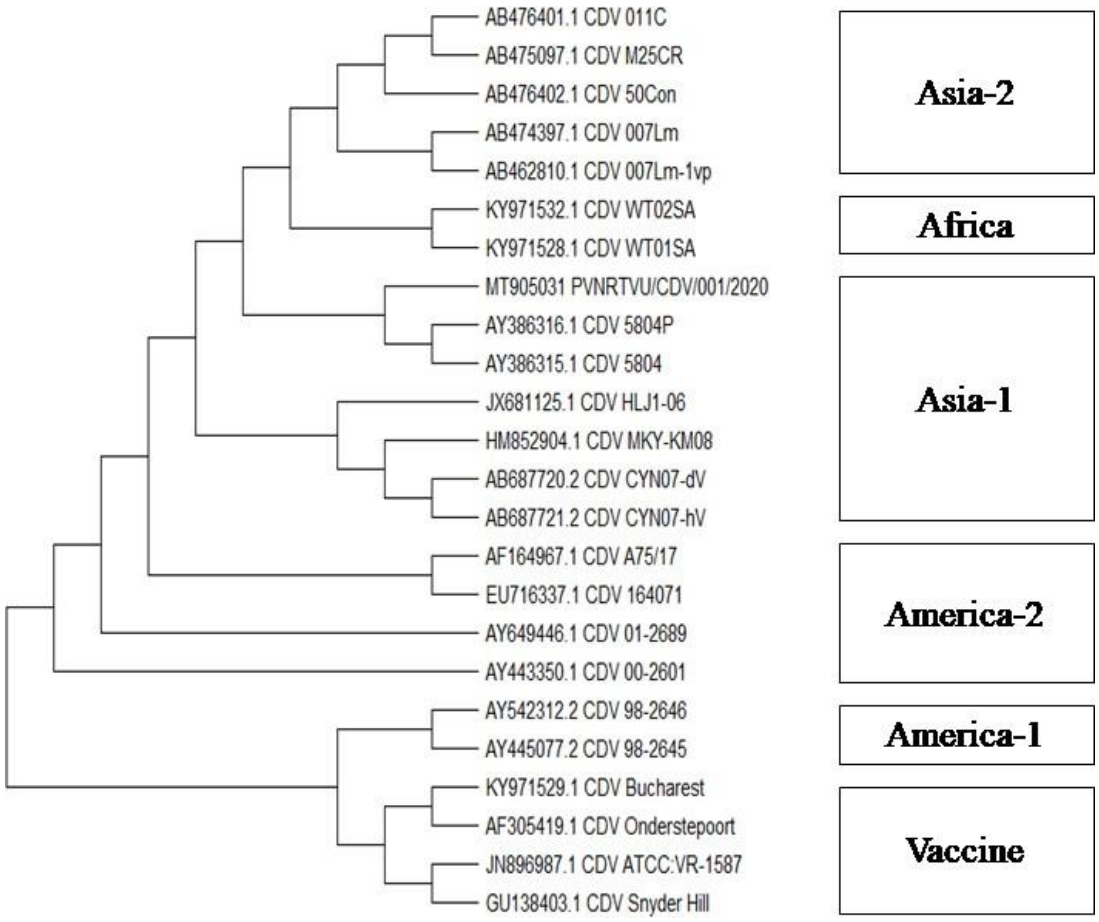


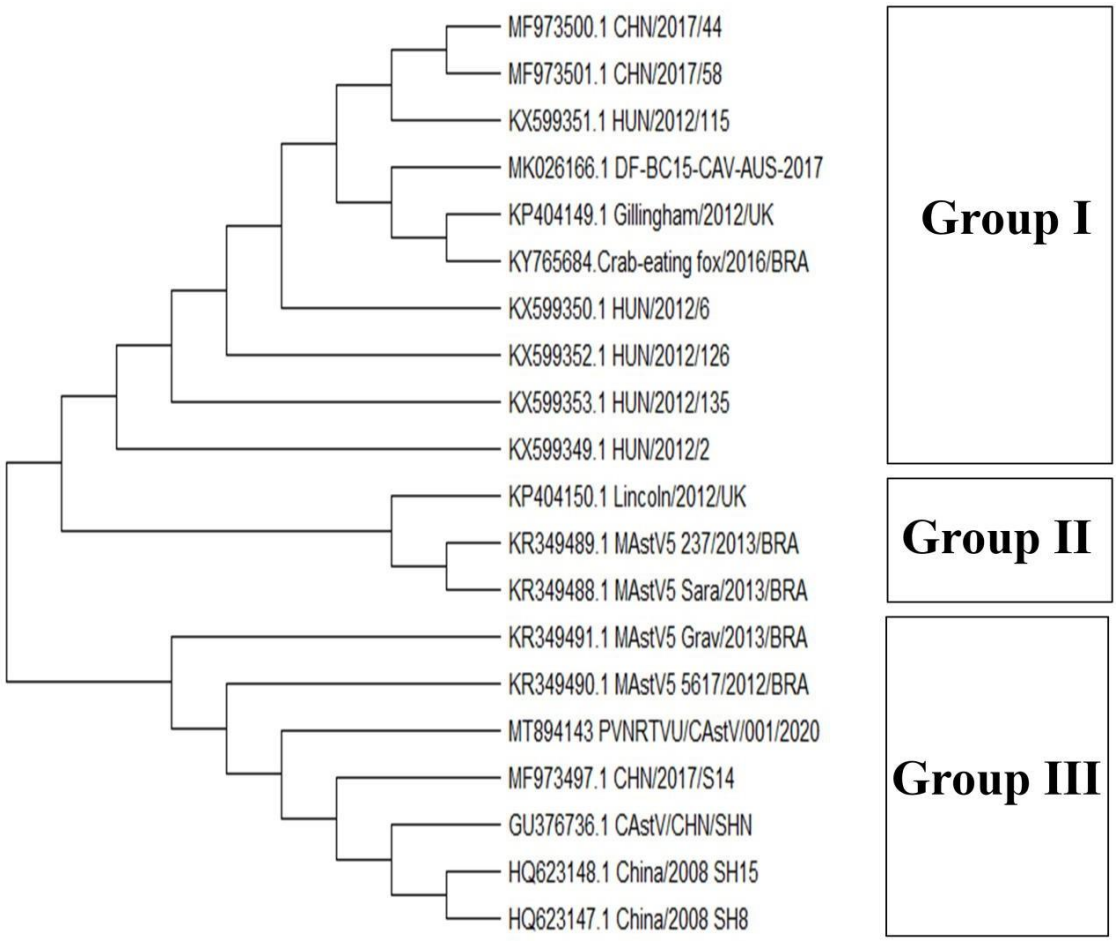
Fig 4.15 Phylogenetic analysis of L-geneof CDV

The phylogenetic tree was constructed by using the maximum likelihood method on Tamura Nei model. The tree shows the relationship between the current isolate MT905031 PVNRTVU/CDV/001/2020 and other isolates from different countries. The current isolate clustered into the ASIA-1 lineage and all the vaccines clustered into separate lineage distinct from the current isolate.

PHYLOGENETIC ANALYSIS OF CAstV

Whole genome sequence of CAstVisolated in the current study (PVNRTVU/CAstV/001/2020) was compared with the CAstV sequences belonging to different geographical regions obtained from the GenBank like CHN/2017/44 (GenBank Acc. No. MF973500.1) CHN/2017/58(GenBank Acc. No. MF973501.1) HUN/2012/115 (GenBank Acc. No.KX599351.1) CAV-AUS-2017 (GenBank Acc. No. MK026166.1) KP404149 (GenBank Acc. No. KP404149.1) crab eating fox/2016/BRA (GenBank Acc. No. KY765684.1)HUN/2012/6 (GenBank Acc. No. KX599350.1) HUN/2012/126 (GenBank Acc. No. KX599352.1) HUN/2012/135 (GenBank Acc. No. KX599353.1) HUN/2012/2 (GenBank Acc. No. KX599349.1) Lincoln/2012/UK (GenBank Acc. No. KP404150.1) MAstV5 Grav/2013/BRA (GenBank Acc. No. KR349491.1) MAstV5 Sara/2013/BRA (GenBank Acc. No. KR349488.1) MAstV5 237/2013/BRA (GenBank Acc. No.KR349489.1) MAstV5 5617/2012/BRA (GenBank Acc. No. KR349490.1) CHN/2017/S14 (GenBank Acc. No. MF973497.1) CHN/2008/SH8 (GenBank Acc. No. HQ623147.1)CHN/2008/SH15 (GenBank Acc. No. HQ623147.1) CHN/CAstV/SHN (GenBank Acc. No. GU376736.1) (Fig.4.16).

Based on the clustering of isolates from different geographical locations, they were divided into three lineages i.e., Group-I, Group-II and Group-III. The current isolate PVNRTVU/CAstV/001/2020 clustered into Group-III (Fig.4.16). Individual gene phylogeny of the current isolate also clustered into Group III lineage. The phylogenetic tree of ORF-1a (Fig.4.17), ORF-1b (Fig.4.18) and ORF-2 (Fig.4.19) are represented below.



4.16Phylogeneticanalysis of CAstV whole genome

The phylogenetic tree was constructed by using the maximum likelihood method on Tamura Nei model. The tree shows the relationship between the current isolate MT894143 PVNRTVU/CAstV/001/2020 and other isolates from different countries. The current isolate clustered into the Group III.

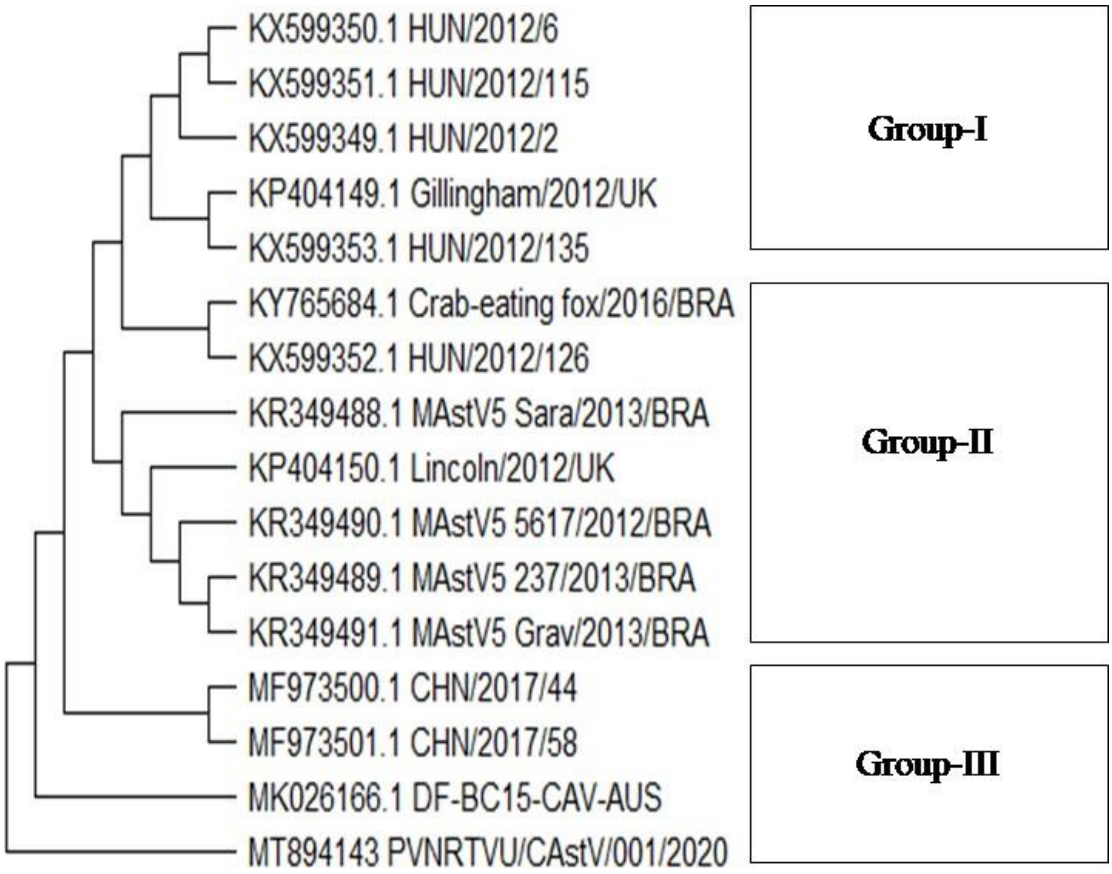


Fig.4.17 Phylogenetic analysis of CAstV ORF-1a

The phylogenetic tree was constructed by using the maximum likelihood method on Tamura Nei model. The tree shows the relationship between the current isolate MT894143 PVNRTVU/CAstV/001/2020 and other isolates from different countries. The current isolate clustered into the Group III.

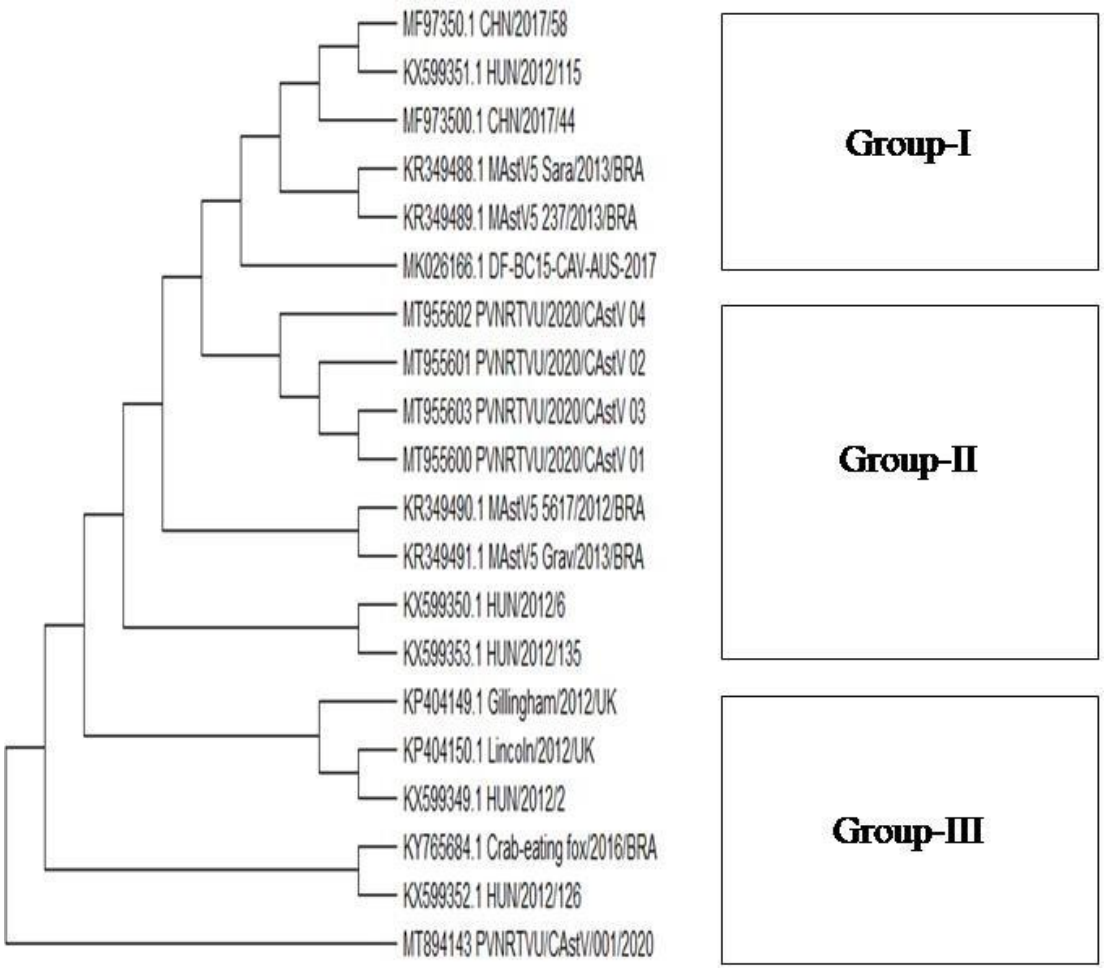


Fig.4.18 Phylogenetic analysis of CAstV ORF-1b

The phylogenetic tree was constructed by using the maximum likelihood method on Tamura Nei model. The tree shows the relationship between the current isolate MT894143 PVNRTVU/CAstV/001/2020 and other isolates from different countries. The current isolates clustered into the Group III and Group II lineages.

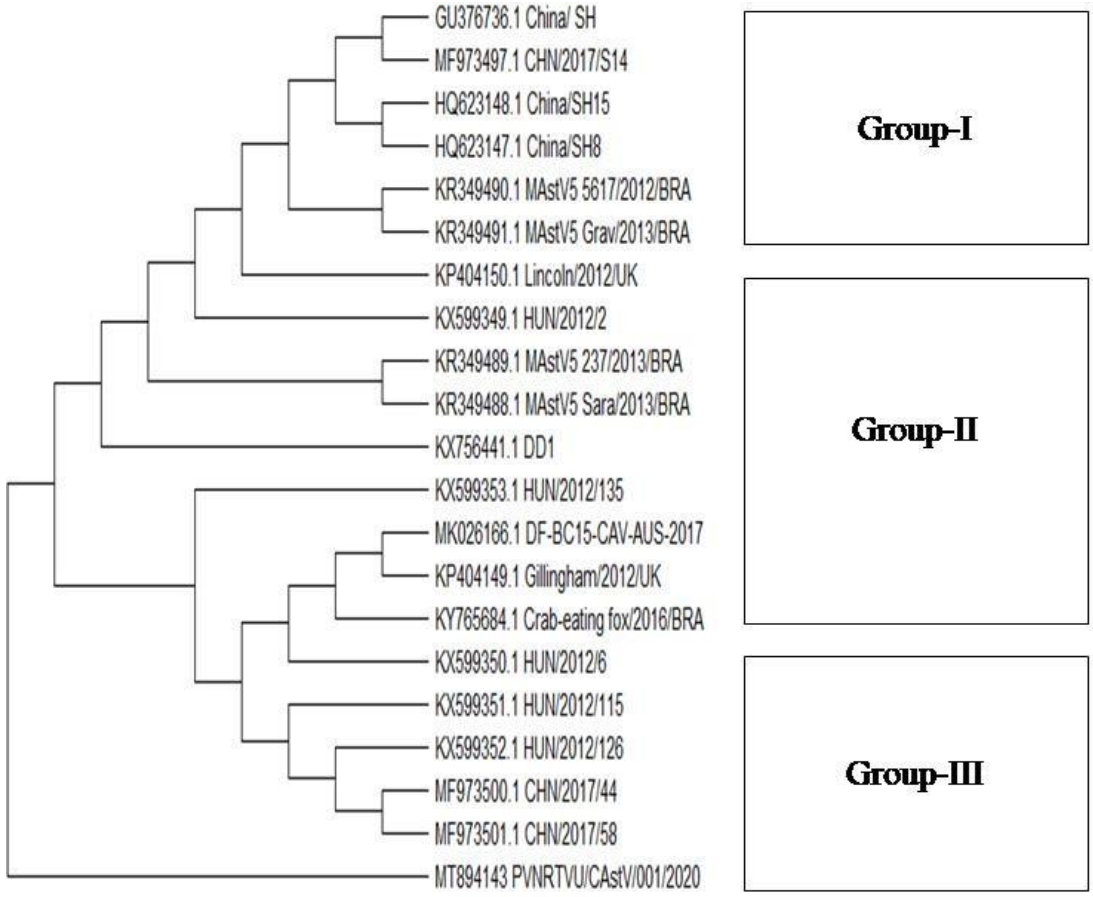


Fig.4.19 Phylogenetic analysis of CAstV ORF-2

The phylogenetic tree was constructed by using the maximum likelihood method on Tamura Nei model. The tree shows the relationship between the current isolate MT894143 PVNRTVU/CAstV/001/2020 and other isolates from different countries. The current isolate clustered into the Group III.

CHAPTER V

DISCUSSION

Gastroenteritis refers to inflammation of the gastrointestinal tract, meaning the stomach and the intestines. Gastrointestinal disorders are frequently reported in companion animals such as dogs as a leading cause for severe dehydration and death. This condition is often characterized by clinical signs such as abdominal pain, diarrhea and/or vomitions. Although there are multiple causes for gastroenteritis in canines, the most common cause is of viral etiology. The viruses associated with enteric illnesses in dogs causes high mortality in non-protected populations (Alves *et al.*, 2018). In this study, the major focus was on viral enteritis caused by RNA viruses such as CAstV and CDV.

CAstV of the genus *Mamastrovirus* (ICTV, 2020) is a non-lipid enveloped, icosahedral, positive-sense, single-stranded RNA virus having a genome size of 6.4–7.3 kb in size (Mendez and Arias, 2007) with three ORFs, a 5′ UTR and a 3′ poly-A tail (King *et al.*, 2011). CDV belongs to the genus *Morbillivirus* within the family *Paramyxoviridae* and order *Mononegavirales* (ICTV, 2020). CDV is an enveloped, single-stranded, non-segmented, negative-sense RNA virus with a diameter of about 150-300 nm in size. The genome of CDV is about 15.7 kb in size and encodes for six structural proteins: N, M, F, H, L, and P (Messling *et al.*, 2001).

The present study was targeted to isolate, characterize and to unravel the molecular evolutionary dynamics of RNA viruses CAstV and CDV causing gastroenteritis in India.

MOLECULAR DETECTION OF CDV AND CAstV IN CLINICAL SAMPLES

PCR is a highly sensitive, rapid, specific technique that can be used for molecular identification of viruses (Frisk *et al.,* 1999). In this study, the samples (N=150) were screened by PCR for CAstV and CDV by using specific primers that target ORF-1b gene and H-gene, respectively. ORF-1b gene is highly conserved nature and was widely used for the molecular detection of CAstV (Vinje *et al.,* 2003). The H-gene is used to differentiate between the wild type and vaccine type strains of CDV (Sheshberadaran *et al.,* 1986; Moller *et al.,* 1992 and Pardo *et al.,* 2005). The PCR was performed using previously published primers for detection of CAstV (Martella *et al.,* 2011) and for CDV (Ashmi *et al.,* 2017).

Of the 150 samples screened, the rate of morbidity for CDV was 4%. Similarly, Agnihotri *et al.* (2017) previously reported 2% morbidity from gastroenteritis cases in dogs and Ashmi *et al.* (2017) reported 2.2% morbidity from CDV suspected dogs using ocular and nasal swabs.

Of the 150 samples screened, the rate of morbidity for CAstV was 3.3%. However, Zhou *et al*. (2017) previously reported 24.3% morbidity, Li *et al.* (2018) reported 39.3% morbidity, Takano *et al.* (2015) reported 9.6% morbidity, and Martella *et al.* (2011) reported 24.5% morbidity in molecular detection of CAstV from the cases of gasteroenteritis. The morbidity reported in this study was significantly lower than the previous reports. The lower rate of infection in this study might be due to the age difference (1m- 5years) of the dogs from which the samples were collected wherein previous reports show samples were collected from 3 months old puppies only. However, in this study, CAstV was detected in puppies of age group 1.5- 5 months.

PROPAGATION OF CDV AND CAstV

There were very limited reports on isolation of CDV and none for CAstV from the clinical samples from India. It is a well-established fact that isolation of virus is tedious and difficult process (Swathi *et al.,* 2016). Attempts were made to isolate CDV and CAstV by passaging the fecal samples in B95a (Kai *et al.,* 1993) and MDCK cells (Martella *et al.,* 2011), respectively.

Of the five suspected fecal samples cultured, only two showed a characteristic CPE for CDV such as rounding of cells and ballooning. CPE was observed in only first passage. Further studies are required to elucidate on why there is no evidence of CPE upon subsequent passages. Similar to this finding, Haritha *et al.* (2020) also noticed the CPE in only first passage when inoculated in B-95a cell lines whereas Ashmi *et al*. (2017) could only recover the CDV from the third passage onwards when passaged in MDCK cells and Dog SLAM expressing Vero cells.

All the six fecal samples of CAstV showed CPE such as rounding of cells and ballooning when inoculated in MDCK cell line, but only one isolate was confirmed as positive by PCR. The reason for this is largely unknown but it can be hypothesized that the CPE shown by other samples might be due to the predominant growth of CPV, the predominant gastroenteritis causing virus. To verify this, the samples were screened for CPV and were found to be positive for CPV-2. This strengthens the assumption that MDCK cells are common for both CPV and CAstV isolation. Hence, it was concluded that the samples of mixed infection show predominantly CPV in cell culture due to its highly virulent nature (Decaro and Greene, 2012).

Martella *et al.* (2011) isolated CAstV from fecal samples of dogs with diarrhea in MDCK cells which triggered a clear cytopathic effect consisting of enlargement and/or detaching of cells and appearance of fine granules in the cytoplasm.

WHOLE GENOME CHARACTERISATION

Nucleotide Sequencing

In order to understand the molecular characteristics such as nucleotide and amino acid variations in the current isolates with that of the reference isolate, PCR products of partial H gene (449 bp) of CDV and ORF-1b gene (295bp) of CAstV were sequenced at sequencing facilities, Bioserve Biotechnologies India Pvt Ltd, Hyderabad using respective forward primers.

The partial-H gene sequence of the current CDV isolates were compared against reference and vaccine strains of CDV isolates from GenBank database and found 91-95% nucleotide identity and 87-95% amino acid identity. Previous reports suggest that the H gene is highly variable with respect to amino acid sequence (Martella *et al*., 2006 and Sarute *et al*., 2013). Hence, it was hypothesized that the variation in the current CDV isolates might be due to genetic variability in the H gene region. Previously it was reported that the NdeI restriction site was found only in wild type isolates leaving out the vaccine strains (Hashimoto *et al.,* 2001 and Calderon *et al*., 2007). Multiple sequence alignment of partial H gene of the current isolates revealed that there is NdeI restriction site, which indicates that it is of field type (Fig.4.8).

The nucleotide sequence of ORF-1b gene of CAstV isolates were compared against reference strains which revealed that there is 97-99% nucleotide identity and 97-100% amino acid identity with each other and 91-100% nucleotide identity with other sequences in GenBank database. Li *et al.* (2018) found high range of variation in the partial ORF-1b nucleotide sequence identity of 77.6–97.4%. Likewise, Zhou *et al.* (2017) found a wide range of genetic diversity i.e., 77.7-100% of amino acid identity for the partial ORF-1b gene.

Phylogenetic Analysis of CDV

In order to understand the genetic variations across the genome, the whole genome sequencing was performed at MedGenome Labs Ltd. Bengaluru. To understand the molecular evolutionary relationship, the phylogenetic tree was reconstructed using Maximum Likelihood method in MEGA7.0 software by setting the test of phylogeny as bootstrap method and no. of replicates as 1000. The evolutionary distances were computed using the Tamura-Nei model. The clades were divided according to geographical distribution or as per relativeness in to different lineages.

The phylogenetic tree was reconstructed and found that current CDV isolate (GenBank Acc No. MT905031) belong to Asia-1 lineage. All the vaccine variants clustered under one lineage. Similarly, Romanutti *et al.* (2020) found that their isolate clustered into South America-2 lineage whereas all the vaccine strains clustered into separate lineage based on whole genome analysis. However, no reports were found on the whole genome sequence of CDV from India.

Earlier the phylogeny trees were majorly reported on the individual genes such as H-gene, F-gene, L-gene, N-gene and P-gene. Therefore, phylogenetic analysis of these individual genes will generate information on the circulating variants of CDV. So, in this study, the individual gene phylogenetic trees were reconstructed using the whole genome sequence and found that N-gene, M-gene, F-gene and L-gene clustered into the Asia-1 lineage whereas P-gene clustered into Asia-2 lineage and H-gene clustered with American strains. All the vaccine variants clustered under vaccine lineage.

Similarly, Tong *et al.* (2015) constructed phylogenetic trees of N and F genes CDV isolated from Chinese raccoon dog and reported that N and F genes clustered into Asia-1 lineage and all the vaccine strains clustered into a separate lineage.

Swathi *et al.* (2016) reconstructed the phylogenetic tree of CDV based on partial-H and L-gene and reported that partial-H gene clustered into American lineage and L-gene clustered into separate lineage closely related to Chinese strains clearly distinct from vaccine lineage.

Ashmi *et al.* (2017) reconstructed the phylogenetic tree of CDV based on H and F-gene and reported that the field isolates of their study clustered into a separate lineage clearly distinct from the vaccine lineage.

Haritha *et al.* (2020) constructed phylogenetic tree of CDV isolate partial-H gene and reported that their isolate clustered into Asia lineage clearly distinct from vaccine lineage.

Based on these reports, there was no change in the lineages formed but internal shuffling of lineages was found which might be due the genetic diversity of the isolated strains. All the vaccine strains clustered into separate lineage indicating the significant difference between the wild and the vaccine strains. Hence, there is an immediate need to emphasize replacement of existing strain with circulating variants of CDV.

Phylogenetic Analysis of CAstV

To understand the molecular evolutionary relationship, the phylogenetic tree was reconstructed using Maximum Likelihood method in MEGA7.0 software by setting the test of phylogeny as bootstrap method and no. of replicates as 1000. The evolutionary distances were computed using the Tamura-Nei model. The clades were divided according to the relativeness into different groups as reported previously.

The phylogenetic tree was reconstructed and found that current CAstV isolate (GenBank Acc No. MT894143) clustered with Group-III lineage that consists of China and Brazil isolates when divided into three groups based on the relatedness. However, only limited reports were found on phylogenetic analysis of CAstV whole genome based on geographical distribution (Caddy and Goodfellow, 2015). Caddy and Goodfellow. (2015) reported a similar observation for CAstV where one of the strains they sequenced got clustered with China isolates. Li *et al.* (2018) reported a similar observation for CAstV in China where one of the strains they sequenced was closely related to the Hungary and UK strains based on the whole genome sequence.

Previous reports on phylogenetic analysis of CAstV were based on the individual genes. So, in this study, the individual gene phylogenetic trees were reconstructed using the whole gene sequences and it was found that ORF-1a clustered with Chinese isolates, ORF-1b clustered with Brazilian isolates and ORF-2 branched sseperately from the existing lineages. This suggests an independent evolution of individual genes.

Several studies reported phylogenies of individual CAstV genes. Choi *et al.* (2014) reported that the Korean strains of CAstV clustered with Group-II lineage that consists of Italy and France strains based on partial ORF-2 gene. Zhou *et al.* (2017) reported that phylogenetic analysis of CAstV based on partial-ORF-1b gene of the strains they sequenced clustered with *Mammastrovirus-5* and the strains were closely related to Italy and Chinese strains based on ORF-2 gene. Li *et al.* (2018) also reconstructed the phylogenetic tree based on individual genes (ORF-1a, ORF-1b, ORF-2) and reported that the CAstV strain they sequenced was closely related to the Hungary and UK strains. Alves *et al.* (2018) reported that the Australian strains of CAstV were closely related to European strains based on partial ORF-2 gene. Bhatta *et al.* (2019) reported that the Brazilian strains of CAstV were closely related to Chinese strains based on partial ORF-2 gene. Zhang *et al.* (2020) reported that the strains of CAstV they sequenced clustered with lineages 2, 3 and 4 that were closely related to Chinese strains based on ORF-2 gene and ORF-1b gene of CAstV were closely related to California sea lion astroviruses.

These reports suggest that the CAstV belonging to different geographical locations clustered into different lineages which might be due to the independent evolution of genes due to various host selection pressures. Due to the presence of only

limited genomic data, it poses a challenge to understand the true genetic diversity and evolution of this virus. Overall, the detailed genetic evolution and diversity analysis of CAstV reveals epidemiologic features that can guide future preventive and control measures. However, no reports were found on the whole genome phylogeny of CAstV from India and this is the first report.

According to the results from the above studies, following conclusions are drawn.

1. PCR due to its specificity, rapidity and sensitivity can be used as a potential diagnostic technique for detection of viruses.
2. The H-gene of CDV is more prone to genetic variation and there is significant difference between the wild type and vaccine strains. Phylogenetic analysis of the current isolate against other available sequences revealed that all the vaccine strains clustered into one lineage. This shows that there should be an immediate emphasis on development of vaccines against circulating strains of CDV and installing proper vaccination measures for effective control of CDV.
3. CAstV also plays a major role in causing gastroenteritis. Phylogenetic analysis of the current isolate revealed that they clustered into Group-III lineage. It also exists in genetically variant forms at any point of time. Hence, there is an immediate requirement to emphasize on development of a multivalent vaccine.
4. We report the whole genome sequences of CDV and CAstV for the first time in India.
5. This is also the first study on CAstV prevalence and its role in canine gastroenteritis, in India.

CHAPTER VI

SUMMARY

Dogs are prone to several bacterial and viral diseases, most of which cause gastroenteritis. Gastroenteritis refers to inflammation of the gastrointestinal tract, meaning the stomach and the intestines. There may be several causative agents for gastroenteritis such as viruses, bacteria, or protozoans; of which viral etiology is of utmost importance. Viruses associated with enteric illnesses in dogs are an important cause of mortality in dogs. In this study, the major focus was on viral enteritis caused by RNA viruses such as CAstV and CDV.

CAstV which is the causative agent of gastroenteritis in pet dogs was first identified in the early 1980s. However, it has been reported in the feces of dogs with and without diarrhea. CAstV of genus *Mamastrovirus* is a non-lipid enveloped, icosahedral, positive-sense, single-stranded RNA virus having a genome of 6.4–7.3 kb in size with three ORFs, 5′ UTR and a 3′ poly-A tail. ORF1a and ORF1b located at the 5′ end of the genome that encodes for non-structural proteins. ORF2 encodes a capsid protein, which is located at the 3′-terminal end.

CDV belongs to the genus *Morbillivirus* within the family *Paramyxoviridae* and order *Mononegavirales.* CDV is a single-stranded, negative-sense, non-segmented, enveloped RNA virus with a diameter of about 150-300 nm in size. The genome size of CDV is about 15.7 kb that encodes for six structural proteins: N, M, F, H, L and P. The present study was targeted to isolate, characterize and to unravel the molecular evolutionary dynamics of RNA viruses, CAstV and CDV causing gastroenteritis in India.

A total of 150 fecal samples were collected from the dogs exhibiting gastroenteritis, presented to various private clinics in Hyderabad and Veterinary

Clinical Complex, C.V.Sc, Hyderabad were utilised for detection, isolation and molecular characterisation of CDV and CAstV.

The fecal samples were processed, RNA was extracted and cDNA was synthesized to use it as a template for PCR. The PCR was performed by gene-specific primers targeting ORF-1b gene and partial-H gene respectively. Six samples were found positive for CAstV (4%) and five samples were found positive for CDV (3.3%). Two out of five cultured samples showed characteristic CPE in B-95a cells for CDV and confirmed as CDV. All the six cultured samples showed a characteristic CPE in MDCK cells for CAstV, one was confirmed positive by PCR.

Nucleotide sequencing of ORF-1b gene of CAstV PVNRTVU/2020/CAstV 01-04 showed 97-99% nucleotide identity with each other. Nucleotide sequencing of partial-H gene of CDV isolate with reference to vaccine strains and other sequences of CDV isolates from GenBank database showed 91-95% identity. Multiple sequence alignment of partial H gene revealed that there is *NdeI* restriction site in field isolates.

Further, the whole genome sequencing was done for CDV and CAstV for the first time in India. Upon phylogenetic analysis, the current CDV isolate clustered into Asia-I lineage and CAstV clustered into Group-III lineage.

From the present study, it may be concluded that there is an immediate need to emphasize on the development of the multivalent vaccine with the current circulating strains of CDV. The present study also revealed that CAstV have a significant role in canine gasteroenteritis, suggesting the need of extensive studies throughout the country on the prevalence of CAstV for effective control measures.

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