MOLECULAR PROFILE OF CANINE PARVO VIRUS

T H E S I S

Submitted

In partial fulfillment of the requirements for the Degree of

MASTER OF VETERINARY SCIENCE

IN

VETERINARY MICROBIOLOGY

BY

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Enrollment No: V/14/179

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(INDIA)

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DECLARATION OF STUDENT

I hereby declare that the experimental research work and interpretation of the thesis entitled "**MOLECULAR PROFILE OF CANINE PARVO VIRUS"** or part thereof has not been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis / publication of any University or scientific organization. The sources of materials used, and all assistance received during the course of investigation have been duly acknowledged.

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To My Beloved

Parents

Mr. Nagnath Panchal

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Mrs. Godavari Panchal

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Place: Parbhani *(Dr. Panchal Mahesh Nagnath)*

Date: / /

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LIST OF ABBREVIATIONS

AGE	:	Agarose gel electrophoresis
Bp	:	Base pair
cDNA	:	Complementary deoxyribose nucleic acid
DW	:	Distilled water
et al.	:	Italia (and others)
Fig.	:	Figure
IU	:	International units
Mg	:	Milligram
MgCl2	:	Magnesium chloride
Min	:	Minutes
NFW	:	Nuclease free water
ng	:	Nano gram
nm	:	Nanometres
^{o}C	:	Degree Centigrade (Celsius)
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
RNA	:	Ribonucleic acid
µg	:	Microgram
µl	:	Microliter
Cacl2	:	Calcium chloride
ND	:	Non-Descript
V	:	Vaccinated
NV	:	Non Vaccinated
CPV	:	Canine Parvovirus

Introduction

CHAPTER - I

INTRODUCTION

Dog have secured a special place in man's life as human companion who provides an embodiment of love and affection. Dogs have diversified roles from pets to sporting companions who are helpful to mankind in stress reduction, health benefits, security, other services like tracking, hunting, instruments of war, bomb detecting squad and healer of both physical and emotional problems of humans, detecting criminals and guiding blind people. Canine parvovirus (CPV) is one of the most important viral cause of acute canine enteritis that to severe damage of the intestinal barrier. It has been speculated that dogs might develop chronic disorders after surviving CPV infection (Kilian *et al*., 2018). Canine parvo virus (CPV) infection due to its highly infectious nature, high morbidity and mortality rates has raised concern not only to pet owners but also practicing veterinarians and scientists. Most of the parts of the world have recorded the reports of CPV infection including Asia, Australia, New Zealand, the Americas, and Europe. In India 1st outbreak of CPV infection was reported in Madras (1981) and in Bombay (1985) (Hasan *et al.,* 2017). The prevalence study in India was first reported by Balu and Thangaraj in Madras. The pattern of disease experienced in a population is largely influenced by the susceptibility of host, environmental conditions such as housing, hygiene, population density, and pathogenicity of the infectious agent (Behara *et al.,* 2015).

Members of family *Parvoviridae* are small, resilient, non-enveloped viruses with linear, single-stranded DNA genomes of 4–6 kb ICTV (2020).

The canine parvovirus belongs to the *Parvoviridae* family, with the genus Protoparvovirus and the species Carnivore protoparvovirus 1 as its members. It is a non-enveloped virus with a single-stranded DNA genome that encodes two capsid structural proteins, VP1 and VP2, which are required for viral genome assembly and packaging, and two non-structural proteins, NS1 and NS2, which control DNA replication, assembly, and gene expression regulation (Faz *et al*., 2019). Based on the evolutionary modifications, CPV-2 is thought to have

evolved from a feline panleukopenia-like virus that infects wild predators. Due to the mutations in the virus in the subsequent years, two additional antigenic variations, type 2a and type 2b, were discovered. Both variations fully replaced the original type 2 in the canine population and were spread all over the world. Following then, many CPV mutations were discovered in other nations. In Italy, a novel CPV mutant was discovered with an amino acid mutation (Asp-426 to Glu) in a critical position for CPV-2 antigenicity. Adult dogs that were vaccinated with type 2-based formulations also were observed with those variations that were prevalent across all continents and linked to epidemics of hemorrhagic gastroenteritis (Filipov *et al*., 2011). CPV-2 is believed to be derived from the feline panleukopenia virus (FPLV), where specific mutations occurred at Lys80Arg, Lys93Asn, Val103Ala, Asp323Asn, Asn564Ser and Ala568Gly capsid protein VP2 residues that facilitated a change of host, thereby allowed the virus to infect canines and lost the ability to infect felines. These mutations in the CPV-2 variant lead to its spread to other countries like Europe, America, Asia and Oceania (Ramirez *et al*., 2020).

In the year 1982, it was observed that CPV-2 was replaced by a variant of the virus that genetically and antigenically differed named as CPV-2a that differed from CPV-2 in 6 amino acids: Met87Leu, Ile101Thr, Ser297Ala, Ala300Gly, Asp305Tyr and Val555Ile residues (Ramirez *et al*., 2020). The another variant CPV-2b (VP2 protein showed residue 426 that changed from Asn426Asp and then from Asp426Glu in the CPV-2b and CPV-2c antigenic variant strains, respectively. Antigenic variants, CPV-2a and CPV-2b divergence occurred from the CPV-2 strain due to changes in 5–6 amino acids in the antigenically important VP2 capsid region and regained their ability to infect feline and canine host. CPV and FPV are two closely related viruses, causing disease in respective hosts, but new variants of CPVs have acquired the feline host range allowing them to infect both cats and dogs, whereas the original CPV-2 does not replicate in cats (Ahemad *et al.,* 2018). At present, there are four known antigenic types of CPV circulating throughout the world, namely, CPV-2, CPV-2a, CPV-2b, and CPV-2c. The CPV-2c variant which was emerged in Italy. That has also been reported from some parts of India (Nandi *et al.,* 2010a). Currently, these mutants have

replaced the prototype CPV-2a and CPV-2b and co-circulating with prototypes in many countries (Mira *et al*., 2018) including India (Nookala *et al*., 2016).

Carnivore parvovirus known to infect a wide range of host species and are endemic in most domestic and wild carnivore populations. For years, the related group of ‘‘FPV-like" (Feline parvovirus) viruses, which included FPV, viruses from raccoons and arctic foxes (referred to as blue fox parvovirus (BFPV), was prevalent. Domestic and wild cats, lions, tigers, leopards, cougars, lynx, civets, leopard cats, arctic foxes, and raccoons are among the hosts that were infected by FPV-like viruses. CPV has been reported to infect dogs of all age groups, but puppies are most severely affected than adults (Behara *et al.,* 2015). Canine parvoviral enteritis mostly affects pups aged 6 weeks to 6 months (Goddard *et al*., 2008). The morbidity of the virus is 100%, while mortality among puppies without medical treatment reaches 91%, which can be minimize with veterinary medical care (Munoz *et al*., 2021).

CPV infection has been reported to be transmitted by ingestion of CPV-2 that shed in the vomitus or feces of infected animals and oronasal infection was also detected (Kantere *et al*., 2021). In addition to the fecal-oral route, the feces of dogs, pet shops, kennels, breeding equipments, and veterinary clinic were also reported to act as a secondary source of infection among the canine population (Bajehson., 2010). The virus after infection replicates first in oropharyngeal and mesenteric lymph nodes and thymus, and viremia occurs within 1 to 5 days of exposure. CPV-2 targets thereafter the rapidly dividing cells of the intestinal epithelial crypts, bone marrow, epithelium of the tongue, oral cavity, and cardiac myocytes, in addition to lung, spleen, liver, and kidneys (Hoelzer *et al*., 2010). Parvovirus replicates in the host cell, especially the cell nucleus (Goddard *et al*., 2010). In animals older than 4 weeks Canine parvovirus (Parvoviridae types 2a and 2b) have been reported to infect rapidly proliferating cells in the gastrointestinal tract, lymphoid tissue, bone marrow and myocardiocytes where virus multiplication occurs. Due to the failure of mitosis, viral replication causes cell death. All rapidly dividing cell populations are not equally affected which suggest that the virus has a preference for particular organs. CPV has been

documented to cause hemorrhagic diarrhoea, vomiting, severe leukopenia, and immunosuppression. The lymphoid and intestinal cell affections decide the primary determinant of disease severity (Goddard *et al*., 2010).

There are several factors that increase the severity of illness such as lack of protective immunity, intestinal parasites, overcrowding, unsanitary, stressful environmental conditions and nonspecific factors like weaning. The season of the year, sex, and breed were also reported as predisposing factors for the development of CPV enteritis. Insufficient passive or active immunity, geographical region and the presence of co-pathogens (including canine coronavirus and intestinal parasites enhances severity of CPV infection (Brady *et al*., 2012). Mixed breeds sometimes described as being less susceptible to the disease than purebreds (Miranda *et al*., 2015). Doberman, Rottweiler and German shepherd (GS) dogs were more susceptible to Parvovirus infection than other breeds (Ling *et al*., 2012). The incubation period of this virus is 4-5 days (Mia *et al*., 2021).

CPV infection manifests as a severe systemic and possibly life-threatening disease in susceptible dog populations. CPV related gastroenteritis in dogs is characterized by clinical signs such as diarrhoea, vomiting with or without blood, inapatence, lethargy, fever, anaemia and dehydration. CPV is detected in 40-60 percent of diarrhoeic faecal samples thus gastroenteritis caused by CPV is a major clinical problem in dogs. (Kataria *et al*., 2020). The virus is known to disrupt the intestinal enterocyte that results into blunting of the intestinal villi, which causes the clinical signs of vomiting and hemorrhagic diarrhea in addition to nutrient malabsorption and enteric bacterial translocation. CPV has been reported to cause destruction and collapse of thymic cortex and leukocyte precursors in the bone marrow which leads to leukopenia in infected animals. Canine parvovirus type 2 (CPV-2) is one of the most pathogenic viruses in dogs that causes acute hemorrhagic enteritis and myocarditis in dogs (Nandi *et al*., 2010). It is associated with a survival rate as low as 9% in the absence of treatment, and 64% with treatment (Goddard *et al*., 2008).

CPV strains are ubiquitous in the environment, and have been reported to be stable under favourable conditions like no exposure to sunlight and humid environment) for up to 12 months outside the host body (Wojcik *et al*., 2021).CPV is resistant to disinfectant with extremely stable power to stay in the environment for a long period (Mia *et al*., 2021).

Prevention of CPV infection is based on the use of modified live virus (MLV) vaccines, which are able to stimulate both antibody and cell-mediated immune responses, inducing a strong, long-lasting protection against subsequent challenge with virulent viruses (Ford *et al*., 2017). Vaccination is an important and effective method in the strategies to control and prevent canine parvovirus infection in dogs (Nandi *et al*., 2013). Active virus circulation and initial vaccination programmes helped develop herd immunity in animal populations, which greatly reduced mortality and further spreading of the virus. Many of the currently used vaccines are based on the original virus type CPV-2 isolated at the end of the 1970s or early 1980s (Miranda *et al*., 2016). Several studies have shown that certain vaccines based on CPV-2 protect dogs against infection with the new antigenic types, including CPV-2c, under experimental conditions (Wilson *et al*., 2013, Miranda *et al*., 2016). However, immunisation failures including persistence of maternal immunity at the time of vaccination, vaccination of non-responders and circulation of different antigenic variants of the virus in vaccinated dogs have sometimes developed CPV infection and disease (Decaro *et al*., 2020).

There are several other microbial agents that cause infections of the pups, but the rapid morbidity and high mortality in CPV infection warrant an accurate and fast diagnosis of the disease. Clinical diagnosis is often indecisive and several other viral pathogens may produce similar symptoms in animals. At present laboratory diagnosis of CPV is carried out by haemagglutination (HA) test, antigen capture enzyme linked immunosorbent assay (ELISA), rapid immune chromatography test, virus isolation and polymerase chain reaction (PCR), and real time PCR. None of the tests is considered gold standard test and all the tests have their own advantages and limitations in different types of clinical settings

(Desario *et al*., 2005). The latest generation of benchtop DNA sequencing platforms provided an accurate whole-genome sequence (WGS) (Reuter *et al*., 2013). Molecular sequences through phylogenetic analysis helped to improve biological research areas such as comparative genomics, functional prediction, detection of lateral gene transfer or the identification of new micro-organisms (Dereeper *et al*., 2008).

Antigenic types of CPV in various geographical areas are detected by seroprevalence data and sequencing *VP2* gene for the detection of mutations helped in the identification of the possible new emerging CPV strains especially in North India (Singh *et al*., 2021). In order to find out the antigenic variations in CPV types prevalent in Marathwada region of Maharashtra, the study was carried out with following objectives.

1. Molecular detection of Canine Parvovirus from the feces of dogs.
2. Sequencing and Phylogenetic analysis of Canine Parvo virus.

Review of Literature

CHAPTER - II

REVIEW ON LITERATURE

The Canine Parvovirus (CPV-2) has been emerged in dogs throughout the world including Indian subcontinent and is responsible for severely affecting the health of canines. The disease has been recorded among the non-vaccinated as well as vaccinated Dogs. The damage caused by the CPV among dog population is very high considering the high impact of the disease caused by CPV the literature is reviewed.

2.1 Historical perspective of CPV:

The Neutralizing antibodies to Minute Virus of Canines (MVC) in commercial canine distemper/hepatitis serum were detected as early as 1956, , but the first report on MVC which was isolated by Dr Leonard Binn in 1968 from the normal faeces of US Army dogs at Germany, were autonomous dog parvovirus was initially considered to be non-pathogenic (Binn *et al*., 1970). In an experiment on the antibody level in pups after CPV infection it was observed that the pups that were seronegative before CPV inoculation were detected seropositive by HI test on day 4 post CPV infection in some pups, and titers steadily rose to 1:80-1:640 by PID 14 (Carmichel *et al*., 1994) The pathogenicity of the virus in the form of enteritis and diarrhoea in neonatal pups was detected (Harrison *et al*.,1992).

Horiuchi *et al*., (1994) reported that the diseases caused by FPLV and MEV in cats and mink have been recognised for many years, while CPV appeared unexpectedly in the late 1970s. Retrospective serological investigations revealed that until the mid-1970s, no anti-CPV antibody was found in the sera of domestic dogs or wild canine populations, indicated that CPV was a novel disease for dogs. The significant antigenic and genetic similarities between CPV and FPLV/MEV, as well as its rapid emergence, strongly implied that CPV originated through mutation from one of the feline parvovirus subgroup viruses.

Moon *et al.*, (2008) reported that CPV-2a as the leading cause of CPV-2 infection in Korea. Based on the amino acid substitution of the VP2 gene, CPV-2a was further classified into CPV-2a-I through CPV-2a-V in Korea. The most common variations in Korea, according to their latest epidemiological study, were CPV-2a-I and CPV2a-V.

Nandi *et al.,* (2010b) concluded in his research work that in India the vaccines are based on CPV2 strain which were different from the strains isolated from outbreak strains that belonged to CPV 2b.

Nandi *et al.,* (2010c) reported the first occurrence of CPV-2c strain in India from the haemorrhagic gastroenteritis cases of dogs based on the sequence analysis. They emphasized that CPV-2c mutants have been evolved to emerge as pathogens of dogs in India and it also represented the frequency of this type mutant observed in a dog population. The presence of CPV 2c strain in India supported that CPV-2c is reached a worldwide distribution.

Gauri *et al*., (2013) reported the outbreak of CPV-2c in vaccinated dogs in Anand district of Gujarat by PCR and RFLP studies.

Srinivas *et al*., (2013) studied occurrence of canine parvovirus in southern India. They screened 128 samples for CPV and 69 (53.90%) samples were found to be positive by PCR assay using H primers. New CPV-2a (297-Ser→Ala) was found to be the predominant strain prevalent in different cities across five Southern Indian states/ Union territories. There was also enough indication of circulation of New CPV-2b strain from different states of Southern India.

Behera *et al*., (2015) carried out study on canine parvovirus infection in and around Bhubaneswar, Odisha, India. The breed wise incidence rate calculated over positive cases was found to be higher in Deshi/local breeds (34.48%), followed by German shepherd (17.24%), equal incidence in mixed and Labrador retriever (10.34%), Rottweiler and German spitz showed 6.9% each and lower incidences in four breeds (3.45%) such as Dalmatians, Nea politan mastiff, Pug and Great Dane.

Kulkarni *et al*., (2019) conducted a study to type the CPV circulating strain in the Marathwada region of Maharashtra. The faecal samples (*n*=150) were collected from dogs with a history of diarrhoea and vomition were screened for CPV using haemagglutination (HA) test with porcine RBC's.

Miranda *et al*., (2016) reported that CPV was emerged as a new virus in the late 1970s, which infected domestic dogs, and became distributed in the global dog population within 2 years. A few years later, the virus's original type was replaced by a new genetic and antigenic variant, called CPV-2a. Around 1984 and 2000, virus variants with the single change to Asp or Glu in the VP2 residue 426 were detected (sometimes termed CPV-2b and -2c).

Hasan *et al*., (2017) in his review on Scenario of CPV in India stated that in India the first report on the occurrence of CPV-2 was reported dates back to 1982 by Ramadass and Khader. The incidence of CPV-2 variants in dogs were reported therafter from different states viz. Kerala, Orissa, Assam, West Bengal, Tamil Nadu, Pondicherry, Haryana and Uttar Pradesh.

Hasib *et al*., (2021) carried out amplification- refractory mutation system (ARMS-PCR) and observed the presence of all the three known variants, CPV2a, CPV2b, and CPV2c, in the collected samples. They First time recorded and confirmed above strains by molecular analyses in Bangladesh.

2.2 Incidence of Parvovirus :

2.2.1 <u>On the basis of Age and Sex</u>:

Canine parvovirus is disease occurs mostly in 6 day to 6 months age group of dogs.

Tsao *et al.*, (1991) stated that foetal or newborn animals affected with CPV and caused myocarditis, that lead to death from heart failure. CPV attacked lymphoid tissue in elderly animals and reduced the amount of circulating lymphocytes (panleukopenia).

Castro *et al.,* (2010) studied age wise infection of CPV in the young dogs up to 6 months old of and reported that it to be one of the most prevalent viruses

causing acute gastrointestinal clinical symptoms such as vomiting, anorexia, lethargy, and diarrhoea, with or without melena.

Sykes *et al.*, (2014) carried out study on parvoviral enteritis in dogs older than 6 months old found that intact males were twice as likely as females.

Wilson *et al.*, (2014) conducted study on age of dogs under one year of age have the highest chance of getting serious illness. Causes moderate to severe haemorrhagic enteritis, fever, vomiting, and, in severe instances, death. Although all naive animals are particularly susceptible to infection,

Behera *et al*., (2015) carried study on age-wise prevalence over the positive cases revealed the infection being more in the age group of 3-6 months (41.37%), followed by equal incidences in 1-3 months age group and 6-12 months (27.59%), and a low incidence in age groups above 12 months (3.45%). The age-wise distribution pattern was represented. The incidence was predominantly higher in males (86.21%) in comparison to females (13.79%) with respect to total positive cases , whereas the incidence rate over total suspected cases was found to be 35.21% in males and 5.63% in females.

Rincy *et al.,* (2016) in his study revealed that pups below five months of age group (84.00 per cent) showed higher occurrence of CPV enteritis, followed by dogs of five to eight months (64.70 per cent). The dogs above nine months of age (58.33%) showed least occurrence of CPV. They reported that the occurrence of CPV infections was higher in male dogs (80.65%) than in females (60.87%).

Kilian *et al*., (2018) stated that the median age with acute CPV infection in dogs was 12 weeks (range 5 to 357 weeks) and median time of observation was five years (range 1 to 13 years).

Terzungwe *et al*., (2018) observed that the majority of the dogs affected with CPV were less than 6 months old, females, and exotic breed of dogs that were not vaccinated.

Khare *et al*., (2019) reported the overall prevalence of canine parvovirus infection in dogs was as 7.24%. Dogs between 0-3 months of age showed the

highest prevalence 11.9% followed by 3-6 months of age 7.09% and 6-12 months of age 5.31%. Whereas, the lowest prevalence of canine parvovirus was reported in the dogs above 12 months of age. The sex wise prevalence revealed higher prevalence in male (7.91%) as compared to female (6.36%). The maximum prevalence was noticed in non-descript dogs i.e. 12.57% followed by Great dane, German shepherd, Spitz, Dalmatian, Labrador, Doberman and Lhasa apso in which prevalence was found to be 10%, 6.12%, 5.26, 3.82%, 3.61%, 3.57% and 2.77% respectively. During their study the dogs reared on vegetarian diet showed significantly higher prevalence i.e. 8.90% as compared to dogs reared on non-vegetarian diet i.e. 3.38%.

Torre *et al*., (2018) reported that CPV infection occurred in dogs at different age groups. Out of thirty five ten 10/35 (twenty eight point five 28.5%) were 8 weeks' old, two 2/35 (5.7%) were 10 weeks' old, seven 7/35 (twenty percent20%) were 12 weeks' old, five 5/35 (fourteen point three percent 14.3%) were between 14 and 16 weeks' old, and the remaining nine out of thirty five 9/35 (twenty five point seven percent25.7%) were between 20 and 36 weeks' old, Of the total, 17/35 (forty eight point five percent) animals were male and 18/35 (fifty one point five percent51.5%) were females.

Kazerooni *et al*., (2020) stated that dogs more than 6 months of age and intact males develop CPV enteritis more often than intact female dogs. However, older dogs are occasionally affected. Susceptibility rises with a decrease in maternal antibody, intestinal parasitism, or enteric diseases such as *Campylobacter, Salmonella, Giardia*, and *coronavirus* infections.

Suvethika *et al*., (2021) studied that puppies between the ages of weaning and six months are thought to be the most vulnerable.

2.3 Properties of Canine Parvovirus

Canine parvovirus, or CPV, emerged as a deadly threat to dogs in the late 1970s, most likely the result of the direct transfer of feline panleukopenia or a similar virus from domesticated cats. A mutation in CPV that can profoundly alter transferrin receptor (TfR) binding and infectivity of the virus.

2.3.1 Mutation

Mutation generate genetic variation on which random drift and natural selection can act, shaping the genetic structure of CDV populations leading to some fitness compensations between hosts and driving the evolution of specialist and generalist traits in CDV populations (Valencia *et al*., 2019)

Parez *et al*., (2007) detected novel CPV type in 1984, called CPV-2b, due to antigenic variation which is co-circulating with CPV-2a in dog populations worldwide. The antigenic changes found in CPV-2b were the result of a single aa substitution (Asn426Asp) in the capsid's main antigenic region (epitope A). In the year 2000, a new CPV mutant (Glu-426) was discovered in Italy owing to a Glutamate substitution in the identical 426th residue. As with the antigenic variation CPV-2b, the Asn/ Asp426Glu substitution resulted in an antigenic alteration that could be identified using monoclonal antibodies (MAbs) in haemagglutination inhibition experiments (HI). As a result, some authors have designated the Glu-426 mutant as a new type known as CPV2c, a nomenclature that will be utilised throughout this work.

Hong *et al.*, (2007) distinguished some mutation in CPV from the less pathogenic and antigenically unrelated CPV type 1 or minute virus of canines. The new highly pathogenic virus was called CPV type 2 (CPV-2), was identified and the original CPV-2 evolved further and was fully superseded by two versions, CPV-2a and CPV2b, by the mid-1980s. CPV-2a and CPV-2b were co-circulated in varying amounts across canine populations globally during the last 20 years. In Italy, a novel form of CPV (later called CPV-2c) was discovered in 2000 and was extensively disseminated and co-circulated with types 2a and 2b. CPV-2c was also linked to isolated outbreaks in Spain and Vietnam.

Xylouri *et al.*, (2010) noted evolution of canine parvovirus 2 first that appeared in the late 1970s, which was quickly supplanted by two new antigenic variations, CPV type 2a (CPV-2a) and CPV type 2b (CPV-2b), identifiable by monoclonal antibodies and distinguished by amino acid changes in the capsid protein gene. The two novel antigenic variations were found all over the world.

Nandi *et al.*, (2010a) observed different mutations in canine parvovirus 2 (CPV-2) that caused a major disease of domestic and wild canids. Dogs were known to be infected by two different parvoviruses: the pathogenic CPV-2 and CPV-1, also known as the canine minute virus (MVC).

Raj *et al.*, (2010) discovered, mutational variations in two additional antigenic CPV-2a and CPV-2b. CPV-2a and CPV-2b antigenic types varied from the original CPV-2 by at least five or six amino acids in the VP2 capsid protein.

Brindhalakshmi *et al.*, (2016) carried out study on mutation in amino acids. Novel varieties of canine parvovirus, CPV-2a, 2b, and 2c, have also infiltrated the feline host-range that were capable of infecting and replicating in cats, caused illnesses that were indistinguishable from FPV. There were five to six amino acid changes (87, 101, 300, 305, 426, and 555) between CPV-2, CPV-2a, and its variations.

Miranda *et al*., (2016) observed that CPV showed mutation that were related to a virus similar to the long recognized FPV, but was not of cats origin. They noted that CPV-2 arose when it acquired mutations that allowed binding to the transferrin receptor type-1 (TfR) on the surface of canine cells.

Torre *et al*., (2018) revealed three variants of CPV-2 with a prevalence of 57.1% (20/35) for CPV-2a, 8.5% (3/35) for CPV-2b, and 34.3% (12/35) for CPV-2c. They carried out complete sequencing of the *VP2* gene that showed amino acid substitutions in residues 87, 101, 139, 219, 297, 300, 305, 322, 324, 375, 386, 426, 440, and 514 of the three Ecuadorian variants when compared with the original CPV-2 sequence.

Kwan *et al*., (2020) noted that mutation occurred in CPV strains emergence as a result of acquired amino-acid (aa) substitutions. Mutations in capsid residues were observed to be associated with biological and/or antigenic changes. Canine parvoviruses were also identified that contained a residues typical of FPV at capsid (VP2) key positions, representing reverse mutations or residual mutations retained from CPV-2 during adaptation from an FPV-like ancestor. It also suggested evolutionary intermediates between CPV-2 and FPV

were circulating in the field. Similarly, intermediates between CPV-2a-like viruses and CPV-2 were also identified.

Gainor *et al*., (2021) reported that in South America, new CPV-2a, and other mutants of CPV-2a (VP2 Ser297Asn, Phe267Tyr, Tyr324Ile, and Thr440Ala) were found to coexist with CPV-2b and/or CPV-2c variants at various frequencies, emerging as the major strain.

Chen *et al*., (2021) mentioned that the CPV was continuously undergoing genetic evolution, that gave rise to several variants. They investigated the prevalence of Chinese CPV-2 strains collected from 2018 to 2021 and the VP2 gene was sequenced and analyzed. Two variants, new CPV-2a (297Ala, 426Asn) and CPV-2c (426Glu), were identified. They observed that the CPV-2c variant gained an epidemiological advantage over the new CPV-2a variant in China.

Doan *et al*., (2021) conducted a study on mutation of viral protein 2 (VP2) of canine parvovirus (CPV) that exhibited a high degree of genetic and antigenic diversity. They analyzed 88 Vietnamese CPV-VP2 sequences (1755 bp), 34 from their study and 54 from previous studies, and discovered a new sublineage, "new var.", within the lineage CPV-2c-"new", characterized by the mutation 5G/447M, which was restricted to the Vietnamese isolates. The new mutants appeared to have emerged accounted for 65.5% of the total, with strong nodal support (98%), the distinct Vietnamese 2c-"new-var." sublineage (5G/426E/447M) was found to be separate from the 2c-"new" sublineage (5G/426E/447I) within the 2c-(Asia)/Asia-2c lineage. They reported that the amino acid changes in epitopes of VP2 might have led to the generation of sub-variants and affected the antigenicity, immunogenicity, or virulence of the virus which resulted in vaccine failure worldwide.

Gainor *et al*., (2021) reported two non synonymous mutations, one rare (Asp373Asn) and the other uncommon (Ala262Thr), were observed in a few VP2 sequences. They analysed complete VP2 sequences of 32 strains and identified new CPV-2a (CPV-2a with Ser297Ala in VP2) as the predominant CPV-2 on Nevis Island.

2.3.2 Genome Organisation :

Parvovirus genome is linear, monopartite, ss DNA of approximately 5 kb in size. Most of the packaged strands of DNA are minus-sense, but the adeno-associated viruses package equal amounts of plus and minus sense DNA.

Reed *et al*.,(1988) observed that the genomic organization of CPV-N was similar to that of feline parvovirus (FPV) in that there were two major open reading frames (668 and 722 amino acids) in the plus strand (mRNA polarity). Both coding domains were in the same frame, and no significant open reading frames were apparent in any of the other frames of both minus and plus DNA strands. The nucleotide and amino acid homologies of the capsid genes between CPV-N and FPV were 98 and 99%, respectively.

Battilani *et al*., (2001) conducted study on antigenic drift of CPV and reported that the original CPV-2 strain was completely replaced by the newer antigenic types CPV-2a and CPV-2b, which have also extended their host range to include cats The new types of CPV differed from the original type 2 strain in that there were some nucleotide changes (positions 3045, 3685, 3699, 4062 and 4449) in the gene encoding the VP2 coat protein. Sequences important for the determination of antigenic type and for the control of host range were located in the VP2 capsid protein.

Touihri *et al*., (2009) noted that CPV presumably originated as a host range variant from feline panleukopenia virus (FLV) and adapted to new canine host via wild carnivores like minks and foxes.

Sun *et al*., (2017) stated that genome of CPV-2, a member of the Parvovirus genus in the Parvoviridae family, has a tiny non enveloped, icosahedral capsid with roughly 5.2 kb of single-stranded DNA. ORF1 and ORF2 are open reading frames (ORFs) in the CPV genome. ORF1 encodes two nonstructural proteins (NS1 and NS2), whereas ORF2 encodes two structural proteins (NS1 and NS2) (VP1 and VP2). Notably, VP2 is the most abundant capsid protein with antigen-defining sites, and it is critical in determining CPV antigenic characteristics.

Kim *et al*., (2015) reported that the genome of CPV is a non-enveloped single-stranded DNA (ssDNA) virus with a 5.2 kb genome that encodes two structural (VP1 and VP2) and two non-structural (NS1 and NS2) proteins. VP2 is the most abundant capsid protein and serves as an antigenic determinant.

Mira *et al.*, (2018) stated that genome consisted of an approximately 5,000-nucleotide DNA molecule containing two open reading frames (ORFs), ORF1 and ORF2, encoding for two non-structural (NS1 and NS2) and two structural (VP1 and VP2) proteins through alternative splicing of the same mRNAs. VP2 is the major capsid protein, represents the major determinant of parvovirus host range and is subjected to antibody-mediated selection.

Navarro *et al*., (2020) categorised genome of CPV, which are tiny viruses with a diameter of about 25nm, no sheath, an icosahedral capsid consisting of three structural proteins (VP1, VP2, and VP3), and a single-stranded DNA genome of around 5,000 nucleotides. This thread encodes two structural proteins (VP1 and VP2) as well as two non-structural proteins (VP3 and VP4) (NS1 and NS2). The viral capsid is made up of 60 protein subunits (capsomeres), with the VP2 protein accounting for 90% and the VP1 protein accounting for 10%.

ICTV (2020) in their report noted that CPV have 2 major gene cassettes; a non-structural replication initiator gene (NS) located in the 3′ (by convention the "left") half of the negative-sense strand, and a single capsid sequence (VP) located in the right half. The replication initiator protein (NS1, sometimes called the replicase) has an endonuclease domain that combines sequence-specific duplex DNA-binding and site-specific single-strand nicking activity, followed by a superfamily 3 DNA helicase domain, but it does not have polymerase activity, and these viruses rely entirely upon host DNA polymerase(s) to amplify their genomes during productive infection. Most genomes also encode a small number of ancillary proteins in alternate and/or overlapping open reading frames, thus maximally exploiting the available DNA.

2.3.3 Pathophysiology

CPV is spread between dogs via direct contact and indirect contact (fomite). The virus enters the host through the oronasal route after exposure to contaminated feces. Viral replication starts in the lymphoid tissue of the oropharynx and then progresses to the mesenteric lymph nodes, thymus and small intestine. The incubation period between exposure and development of clinical disease ranges from 4 to 14 days (Doyle *et al*., 2021).

Kazerooni *et al*., (2020) observed that genetic mutations in the capsid gene of feline panleukopenia virus (FPV) occurred and consequently expanded its host range to infect dogs. This virus particularly infected and extinguished rapidly dividing cells such as lymphopoietic tissue, bone marrow, and the villus epithelium of small-intestinal crypts. High levels of the virus was reported to be shed in feces 4-7 days post infection, exposure to infective feces was the main source of disease transmission. Factors such as a stressful environment will also increase the risk of severe infection. Different breeds have different susceptibility to parvovirus infection; nevertheless, with an unknown pathophysiology, mixed breeds are known to be less susceptible than pure breeds. Breeds that have been defined to be at great risk of the disease include Rottweiler's, American Pit Bull, Doberman Pinschers, Terriers, English Springer Spaniels, and German Shepherds.

Suvetika *et al*., (2021) stated that needs the host for replication, specifically, cell nucleus and binds itself to the host cell with double-stranded ends of the genome and it replicates only in rapidly dividing cells such as precursor cells of bone marrow.

Arora *et al*., (2021) conducted study on recruitment of DNA damage response (DDR) kinases at the site of DNA damage caused silencing of cyclin-dependent kinases (CDKs) and cell cycle arrest, paving way for the removal of damaged cells through apoptosis. Depending on host cells and type of virus, Parvoviruses arrested cells at different phases. CPV caused DNA disruption and interfered with the cell cycle, generating time to increase viral progenies that lead to pathological consequences of infection, causing cell death after cell cycle arrest

Mia *et al*., (2021) stated that virus comes in contact with the dog the virus entered into a body. A few days later, significant masses of viruses in the bloodstream are discharged, and the pathogen invades 3-4 days later in bone marrow (fast dividing cell). Then the virus delicates the intestinal cells and formed broad inclusion bodies (eosinophilic intranuclear) by multiplying viruses. After that, privileged the bone marrow, the pathogen abolishes the new cells of the defence system. Alongside, it commences to knock out the best defensive mechanism of the body. Afterward, the pathogen causes the vastest shocking consequences in the gastrointestinal tract, and due to inflammation of the bone marrow, CPV infection is signalized by a reduction in white blood cells. The typical intestine has small finger-like protuberances called villi. These tiny fingers are immensely strengthened in the surface areas that make it difficult to access liquid and nutrient absorption in the gastrointestinal tract.

2.3.4 Virus Multiplication (DNA):

Horiuchi *et al*., (1994) conducted study on multiplication of virus among the feline parvoviruses in the genus Parvovirus, host range variations include feline panleukopenia virus (FPLV), mink enteritis virus (MEV), and canine parvovirus (CPV).

Perez *et al*., (2007) noted that Canine parvovirus type 2 (CPV-2) as an autonomous reproducible virus of the Parvoviridae family with a single-stranded negative-polarity DNA genome. Its 5.2 kb DNA has two open reading frames that encode viral proteins that are nonstructural (NS) and structural (VP). The VP proteins are synthesised from alternatively spliced mRNA, and the VP2 sequence is entirely contained inside VP1. VP2 is mostly composed of the CPV's non enveloped icosahedral capsid, and only a few amino acid (aa) changes in its sequence affect the virus's major biological properties.

Miranda *et al*., (2016) reported that genetic and antigenic changes in the variants were correlated with changes in their host range; in particular, in the ability to replicate in cats and also host range differences in canine and other tissue culture cells. CPV-2 variants have been circulating among wild carnivores and have been well-documented in several countries around the world.

Majumder *et al*., (2018) stated that when DNA viruses enter the nucleus they must locate to sites suitable to sustain replication. Small DNA viruses, such as parvoviruses, required multiple cellular factors for the expression and replication of their genomes. DNA viruses set up replication centres essentially randomly, and factors necessary for replication are recruited to these sites. In their study they observed that DNA viruses initially located to cellular sites that maintained factors necessary for virus replication.

2.3.5 Transmission

Canine parvovirus is usually spread to a dog after the virus enters the dog's mouth, spreads to the lymph and blood vessels and then moves throughout the body. It can take between 4 to 14 days for disease to develop after infection. The virus attacks the cells of the dog's bone marrow and the intestine. Infected dogs generally become severely immunosuppressed, allowing other bacteria, viruses and parasites to proliferate and worsen disease. Infected dogs develop profuse, often bloody, diarrhea.

Tsao *et al.*, (1991) noted that natural CPV transmission occurred largely through the fecal-oral pathway. Affection of CPV to small intestine's growing epithelial cells were also infected, which resulted into diarrhoea and vomiting.

Miranda *et al*., (2016) stated that transmission between domestic and wild carnivores readily occurred, while direct transmission through close contact or predation on smaller carnivores. The viruses were readily transmitted across long distances was probably by fomites.

Khatri *et al*., (2017) stated that the transmission of CPV2 infection spreads mainly through fecal-oral route. The sources of infection were the feces of infected dogs, in dog shows, kennels, parks, and animal shelters. CPV spread very easily to susceptible puppies. They observed that housed dogs were having less chance to exposure to CPV2 than wild dogs. CPV2 was transmitted easily through contaminated objects like utensils, shoes and fomites. Feces contaminated places like veterinary hospitals, pet shops and kennels spread virus to susceptible dogs.

CPV2 is highly resistant to disinfectants and become extremely stable in environment for a long period.

Behdenna *et al*., (2019) in their study on cross-species transmission of CPV reported that it occurred in one direction, from dogs to lions, and that peaks of CPV incidence in lions followed those in dogs were possible transmission routes of CPV. They stated that in addition to faecal–oral route of transmission from fresh faeces, virus could be transmitted after some time from the environment. However, the lack of stable endemic infection, as evidenced by periodic peaks of infection in dogs and periods when CPV infection was absent in lions, as well as the coupling of infection in lions with infection in dogs were not entirely consistent with long-term environmental persistence.

Kelman *et al*., (2020) stated that transmission in Peri-urban regions in Australia were major locations that detected the presence of CPV in wild dogs, as there was close contact between human settlements and wild carnivore populations, which might be associated with exposure to CPV.

Qubaa *et al*., (2021) stated that, oronasal exposure to contaminated urine, hair coats, and fomites such as tools, mosquitoes, and rodents aids in disease transmission. The virus may survive in the atmosphere for months or even years.

2.4 Faecal Sample collection and Processing

Detection of canine parvovirus need to process for PCR. Performing PCR collection fecal sample required. Rectal swab collection is common method.

Decaro *et al*., (2007) collected faecal samples that were homogenized (10% w/v) in phosphate-buffered saline (pH 7.2) and subsequently clarified by centrifuging at 1,500× *g* for 15 min.

Nandi *et al*., (2010) collected rectal swab from 13 dogs suspected of CPV infection. Those swabs were suspended in hanks balanced salt solution (HBSS) (ratio 1:9) and filtered through a disposable syring filters (0.45µ) and then centrifuge at 10000 rpm at 4^0 C for 15 min in a refrigerated centrifuge. The supernatant was carefully pipetted out and stored at -20^0C for further use.

Kulkarni *et al*., (2019) collected faecal samples (*n*=150) from six different locations of western India during the year 2014–15. The dogs between 1 and 18 months of age showed clinical signs of illness like foul smelling watery diarrhoea, red colored faeces, vomition, anorexia, high temperature, depression, rough coat were considered for sample collection. The samples were collected in pre sterilized containers and transported on ice. The samples were preserved at -20 °C until further use.

Rincy *et al*., (2016) collected Fifty-four feacal diarrheic dogs samples suspected for canine parvoviral infection with profuse diarrhea with fetid odour and the blood mixed fecal samples. They recorded the case history with respect to the age, breed and sex. Sterile rectal swabs were used for collection of samples and after collection, the swabs were immersed in 1.5 mL of sterile PBS of pH 7.2.

Dorlikar *et al*., (2019) collected samples by using sterile swabs immersed in PBS and stored at -20°C for further processed. The samples were then centrifuged at 2500 rpm for 20 min at 40°C. The supernatant was separated and preserved at –20°C and was used for further laboratory analyses.

Puvarajan *et al*., (2021) collected rectal swabs from 168 dogs suspected for CPV with haemorragic enteritis. The blood tinged faecal samples were aseptically collected by using sterile swabs in phosphate buffer saline (PBS, pH 7.2) (Hi-Media, Mumbai) and stored in -86° C. The samples of infected dogs collected were homogenised in phosphate buffer saline (PBS, pH7.2) and homogenates (200µL) were frozen and thawed thrice, subsequently clarified by centrifuging at 10000 rpm for 15 minutes.

Manh *et al*., (2021) gathered total of 59 fecal swabs from dogs in Vietnam residing in Hanoi. They recorded essential information of the animals, including male, age, breed, and vaccination status. The swab was immersed in 0.5 mL sterile phosphate buffer saline pH 7.4 and stored at -80^{0}C.

Carrai *et al*., (2021) collected faecal samples from enclosures of mixed cat and dog shelters housing apparently healthy cats without diarrhoea or from healthy free ranging colony cats, and frozen at 20°C or 80°C until processed.

Samples from colony cats were collected from litter trays during hospitalization for neutering. At all shelters sampled, enclosures and litter trays were cleaned at least once per day.

2.5 DNA extraction

Decaro *et al*., (2007) extracted viral DNA from the supernatants of fecal homogenates or from the viral suspensions by boiling for 10 min and chilling on ice. To reduce residual inhibitors of DNA polymerase activity to ineffective concentrations, the DNA extracts were diluted 1:10 in distilled water.

Savi *et al*., (2010) performed viral DNA extraction from fecal sample initially they took 10% feacal sample that was centrifuged at 3000 x g for 5 minutes. An aliquot of 25µl of the supernatant was taken and to this 200µl of 5% chelex resin solutions was added. The contents were mixed and 2 µl of Proteinase K (20 mg/ml) followed by 4 µl of 2M dithiotheriol (DTT) were added, mixed and incubated at 56°C for 45 min. After incubation the mixture was boiled for 8 minutes and vigorously vortexed for 10 seconds and centrifuged at 10,000 x g for 2 minutes (REMI Cooling Compfuge CPR 24). An aliquot of 10µl of supernatant was used for PCR assay.

Zienius *et al*., (2016) extracted DNA from samples of 10% faeces in PBS using the TRIzol method (Invitrogen, Life Technologies, MD, USA). The supernatant containing the DNA was transferred to a new tube and stored at -20^{o}C until it was used in PCR.

Mokhtari *et al*., (2018) collected samples were stored at-20^{0}C until tested. DNA was extracted using tissue and blood DNA extraction kit (DYNABIO, Cat No: KI0015).

Manh *et al*., (2021) studied on viral DNA was extracted using a viral DNA/RNA extraction kit II (Geneaid Biotech, Taiwan). Almost all the dogs (58/59; 98.3%) showed a positive parvovirus infection with the rapid testkit.

Kok *et al*., (2000) extracted DNA by Phenol-chloroform extraction of HSV and yielded the highest absorbance value (0.76) at the endpoint dilution, compared to 0.43 for QIAamp and 0.24 for boiling method(BM-DNA). These

values suggest that phenolchloroform extraction yielded the greater amount of viral DNA, followed by QIAamp and BMDNA.

2.6 Clinical signs:

Robinson *et al*., (1980) reported that puppies died suddenly were between 3 and 8 weeks old. They were either found dead or observed to become restless and dyspnoeic, often crying out before dying within 20 to 30 minutes.

Prittie *et al*., (2004) reported from his study that large fluid and protein losses through the gastrointestinal tract might be resulted in severe dehydration and hypovolemic shock. The Classical signs associated with CPV were impairment in tissue perfusion, including changes in mentation, prolonged capillary refill time, tachycardia, poor pulse quality/hypotension, cool extremities, and low rectal temperature. They noted that the clinical disease was more severe in puppies compromised of humoral immunity, secondary to concomitant infection, low maternal antibody titers, or environmental stresses. Abdominal pain secondary to acute gastroenteritis or intussusception was evident on physical examination.

Gupta *et al*., (2006) noted in CPV infected dogs that caused acute haemorrhagic enteritis and myocarditis. During the acute phase of infection, the virus was shed in faeces (more than 109 virus particles per gm of faeces) from infected dogs, and infected faeces served as the primary source of infection.

Goddard *et al*., (2010) noted that enteritis and myocarditis were the 2 disease entities initially described with CPV-2 infection. CPV-2 myocarditis was very rarely seen, but could be developed from infection in utero or in puppies less than 8 weeks old born to unvaccinated bitches. They also observed that all puppies in a litter that were affected, often were found dead or succumbing within 24 hours after the appearance of clinical signs. The clinical signs included were dyspnea, crying, and retching.

Miranda *et al*., (2016) stated that CPVs have a relatively fast clinical course and killed dogs within 2–3 days after the onset of signs. In enteric form

that comprises vomiting, haemorrhagic diarrhoea, depression, loss of appetite, fever and dehydration.

Pereira *et al*., (2018) observed that CPV was a significant cause of sickness and mortality in some populations, and infectious gastrointestinal disease was prevalent and could be severe in pups. Regardless of the cause, early identification and care were critical to reducing death rates, which were quite high, particularly in young animals.

Mazzaferro *et al*., (2020) found clinical signs of parvoviral enteritis were lethargy, inapatence, vomiting, and diarrhea. The diarrhea varied in appearance from soft to mucoid to liquid and hemorrhagic. Sloughing of the intestinal mucosal lining gave a red gelatinous appearance to the feces. With gastrointestinal fluid losses, interstitial dehydration that progressed to hypovolemic shock. Lack of enterocyte nutrient absorption, systemic bacteraemia, and lack of sufficient hepatic and muscle glycogen stores resulted in significant hypoglycemia with neuroglycopenia and seizures. In addition, systemic inflammation and bacteraemia resulted in septic shock with hypotension and organ failure.

Suvethika *et al*., (2021) reported CPV as a dangerous and contagious viral disease with a high morbidity rate (100%) and recurring death rates of up to 10%. This canine parvovirus infection has two clinical forms: enteritis, that affected dogs of all ages, and myocarditis, which affected puppies younger than three months old. In the early stages, dogs infected with enteritis form exhibited signs of sadness, loss of appetite, vomiting, high fever, and severe diarrhoea.

Qubaa *et al*., (2021) observed that the CPV infected dogs showed various clinical signs, but the salient signs were fever (86%), loss of appetite (81%), and bloody diarrhea (62%), and the associated clinical signs were including the same three above signs together.

2.7 DIAGNOSTIC METHOD FOR CANINE PARVOVIRUS

2.7. Nucleic Acid Based Detection Method

PCR is the most common and accurate nucleic acid based method for diagnosis of CPV that amplifies DNA *in-vitro* by enzymic means at an exponential rate and involves a repeated cycling process (Giasuddin *et al*., 1995).

Molitor *et al*., (1991) stated that PCR method has a potential for application for routine diagnosis of CPV from clinical specimens due to its high degree of sensitivity and specificity. This is especially relevant when concerns exist regarding possible persistence of virus.

Senda *et al*., (1995) noted that PCR is characterized by high sensitivity, specificity, and rapidity and therefore become widely used test for the identification of variable microorganisms. They also reported that with the development of advanced molecular techniques, conventional and real-time PCR assays were gradually developed for CPV-2 detection with higher sensitivity and specificity that showed the best correlation between conventional and real-time PCR for detection of CPV-2. However, real-time PCR did not spread as the primary detection method due to its high equipment and reagent cost.

Filipov *et al*., (2011) extracted CPV DNA and tested by a TaqMan assay, which was able to recognize all CPV strains. Real-time PCR was performed using a commercial real-time detection system, and the data were analyzed with the appropriate sequence detector software. Duplicates of the CPV standard dilutions and DNA templates were simultaneously subjected to real-time analysis.

Sheikh *et al*., (2017) screened eight samples by PCR assay using P1 & P2 primer pairs and expected size was 400 bp. All the samples were positive by CPV- 2ab primer pair and expected size was 681 bp. Also, all samples tested positive for CPV DNA by PCR using CPV-2b specific primers and expected size was 427 bp, indicated that the field sequences were 2b variant.

Sharma *et al*., (2018) isolated DNA by phenol chloroform isoamyl alcohol method for faecal samples. PCR amplification of two VP2 gene specific primers amplified both CPV-2a and CPV-2b types with 681 base pairs (bp)

amplicons size and second primer amplified CPV-2b only with 427 bp amplicons size. The CPV -2b amplicon further differentiated CPV-2b and CPV-2c via RE analysis and sequencing. The 427 bp amplicon (CPV-2b positive) was obtained after PCR and PCR products were purified with PCR purification kit (Gene JET, Thermo, USA). The Purified PCR products contained 50 ng per µl DNA concentration and 260/280 ratio above 1.7 were used analysis.

Al-Hosary *et al*., (2018) tested 49 dogs less than six months old with severe watery to bloody diarrhoea, vomiting, and lethargy for parvovirus infection. The viral antigen was detected serologically using the FASTest PARVO Card Test, and the virus was genetically confirmed using specific primers for both conventional and nested polymerase chain reaction (PCR). Molecular techniques confirmed the infection in 81.63% (40/49) by the conventional PCR and in 97.96% (48/49) by the nested PCR.

Dorlikar *et al*., (2019) performed detection of Parvovirus VP2 gene amplification with CPV-2 ab-F and CPV-2 ab-R set of primers. The extracted viral DNA was amplified by PCR assay using VP2 gene-specific primers. The reaction mixture consisted of 2 µL Template DNA, 10 µL 5X PCR buffer, 3 µL $MgCl_2$, 1 µL dNTPs, 1 µL Taq polymerase, 2 µL Forward Primer, 2 µL Reverse Primer, 29 µL Nuclease-free water to make the volume of 50 µl. All these ingredients mixed properly by vortexing. The PCR was programmed as Initial Denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, annealing at 50°C for 2 min, extension at 72°C for 2 min and a final extension at 72°C for 10 min. 5 µl of PCR product was then mixed with 3 µL of bromophenol blue (6X) and was run on gel electrophoresis and visualized by using UV transilluminator.

Moon *et al*., (2020) detected canine viral agents from 17–81 faecal samples and 17–270 necropsy tissues by PCR. They extracted viral DNA using the QIAamp DNA Mini Kit (QIAGEN) and stored at −20°C. PCR assay kits and specific primers were used to identify canine viral pathogens. CPV, canine distemper virus (CDV), canine para influenza virus (CPIV) and canine

coronavirus (CCoV) were screened using Lilif CPV PCR kit, Lili CDV Nested-PCR kit, Lilif CPIV RT-PCR kit, and Lili CCoV RT-PCR kit (iNtRON Biotechnology). PCR detection of canine parvovirus was performed on samples from dogs. All samples tested positive for CPV-2 and negative for CCoV, CDV, CPIV, CHV, and CAdV-1 and CAdV-2.

Puvarajan *et al*., (2021) standardised rapid method by molecular means for detection of clinically affected Canine Parvo Viral (CPV) infection in various breed of dogs of Cauvery Delta Region, Tamilnadu by polymerase chain reaction (PCR) method. Among 168 haemorrhagic fecal samples suspected for CPV-1 infection collected, 112 were found positive by PCR.(66%) Among different age groups, 154/80,55/67 and 3/21 dogs were positive in pups aged 0-3 weeks, 4–8 and 9–12 weeks respectively. The Labrador breed of dog was found to be the most susceptible breed (n = 43) to parvo viral infection.

Dema *et al.,* (2021) studied a two-step ARMS-PCR for CPV-2 antigenic typing and validated the circulating antigenic variants of CPV-2. They used two pairs of primers (outer forward and reverse primers (OF and OR) & inner forward and reverse primers (IF and IR)) with slight modifications to the previously described strategy so as to perform it as a single-step approach instead of a two-step approach. Result reveals that, 18 samples were positive for CPV-2/2a, 79 samples positive for CPV-2b and 6 samples positive for CPV 2c.

Singh *et al*., (2021) collected 118 rectal swabs from dogs that displayed clinical symptoms of CPV infection and processed for DNA isolation, polymerase chain reaction (PCR) and nested PCR (NPCR). A total of 13 NPCR products were chosen at random for VP2 gene sequence analysis. By PCR and NPCR, the percent positive of CPV was determined to be 28% and 70%, respectively.

2.8 Sequencing and Phylogenetic Analysis:

Parrish *et al*., (1991) carried out a study on phylogenetic relationships between CPV isolates from dogs and the viruses from cats, raccoons, mink, and

arctic foxes. All the CPV isolates clearly formed a single clade, and were most likely derived from a single common ancestral sequence.

Nandi *et al*., (2010) studied the nucleotide sequences of the VP1/VP2 gene of a CPV isolates from India, and the phylogenetic link between the isolate and other CPV isolates was established. By polymerase chain reaction (PCR), 16 of the 36 samples were positive for CPV-2 .The Indian isolate closely resembled a CPV-2b Italian strain, according to phylogenetic analysis based on the nucleotide sequence of the VP-1/VP-2 gene, with 98.4 percent nucleotide sequence homology, indicated very little genetic divergence as it was isolated for the first time in 1978.

Phromnoi *et al*., (2010) constructed phylogenetic and molecular evolution tree of the whole VP2 gene nucleotide sequences of the CPV strains of their study and other sequences from GenBank database with MEGA version 4.0 (http://www.megasoftware.net) using the neighbor-joining method. The reliability of the phylogenetic tree obtained for the VP2 region was evaluated by running 1,000 replicates in the bootstrap test.

Raj *et al*., (2010) reported that Sequence analysis estimated phylogenetic relationship of field virus with the reference strains. They observed that one of the field isolates was phylogenetically closely related to New CPV-2a strains of Japan and India; another field isolates shared ancestral origins with New CPV-2a strains of Korea, USA, Italy, Brazil, Germany, Taiwan and Vietnam; rest other sequences had distinct lineage but shared molecular relationship with New CPV-2a reference strains.

Xu *et al*., (2014) carried out study on sequence and was compared and aligned with the MegAlign program of the DNASTAR multiple program package (DNASTAR Inc., Madison, Wisconsin, USA) using the Clustal method. Multiple sequence alignment was carried out using CLUSTAL W of the MegAlign program, and the phylogenetic tree of complete genes was generated by the maximum likelihood method using MEGA. Bootstrap values were calculated based on 1000 replicates to assess the confidence level of each branch pattern: a bootstrap value of >70% was considered to be significant. Pairwise differences

were calculated using the Jukes–Cantor method. The selective pressures on the CPV were assessed by calculating the non-synonymous (dN) and synonymous (dS) ratio (dS/dN) using the Data monkey web interface (http://www.datamonkey.org/), a maximum-likelihood-based tool for the identification of sites prone to positive or negative selection. The HKY85 nucleotide substitution bias model was chosen for this prediction. Results indicated that the predominant antigenic-type of CPV was CPV-2a.

Balasubramaniam *et al.*, (2017) amplified VP2 gene of CPV by polymerase chain reaction, 10 DNA samples were obtained from faecal materials of haemorrhagic-diarrhoeic dogs near Namkkal, Chennai. Two samples' direct sequences of the capsid protein 2 (VP2) expressing gene revealed three nucleotide differences among isolates from throughout the world. As a result, these two isolates were phylogenetically classified with the CPV 2a type.

Kulkarni *et al*., (2019) extracted CPV DNA from the samples that amplified VP2 gene fragment by PCR. The amplicons were subjected for restriction fragment length polymorphism (RFLP), sequencing and BEAST phylogenetic analysis. The results revealed 6% positivity by PCR. The RFLP results indicated single cleavage site for *ApaL*I and *Hinf*I with an exception of two sites for *Hinf*I. The nucleotide sequences showed non-functional nucleotide changes at different locations. The sequence analysis indicated that the nucleotide divergence within isolates under study was 0.00–0.42%, while the nucleotide homology was 99.58–100%.

Materials and Methods

CHAPTER – III

MATERIALS AND METHODS

3.1 Sample collection

3.1.1 Faecal Sample collection

The current study included dogs aged between 1 to 18 months with a clinical history of diarrhoea and other symptoms like vomition, anorexia, high temperature, anxiety, and rough coat. A total of 25 faecal samples were collected from the dogs that were brought for treatment at Teaching Veterinary Clinical Complex, College of Veterinary and Animal Sciences Parbhani, MAFSU from the different places in and around Parbhani district. The history of the pups was noted along with its age, sex, breed, and vaccination status. Out of twenty five diarrheic fecal samples, 5 were of vaccinated dogs and remaining twenty were of non-vaccinated dogs. All the samples were collected in sterile container that contained phosphate buffer saline (PBS) solution and kept on ice and transferred to -20^0C deep freezer until further processed (Table no 3.1)

3.2 Equipment and lab wares

3.2.1 Micropipette

The micropipettes of different sizes (1ml, 100ml, 200ml Eppendorf, USA) were used.

3.2.2 Micro tips

The micro tips used for DNA extraction and in PCR (Tarson, 10µl, 100µl, 1000µl).

3.2.3 Cliclok micro cetntrifuge tubes

The microcentrifuge tubes used in the centrifugation of DNA samples (Tarsons, 1.5 µl).

3.2.4 Refrigerated Centrifuge

The refrigerated centrifuge (Eppendorf Cooling centrifuge 54170 R) was used for extraction of DNA from fecal samples.

3.2.5 Gel Electrophoresis System

Submarine gel electrophoresis system (GeNei) was used for the electrophoresis of the PCR product and separation of DNA.

TABLE 3.1: Fecal samples of dogs collected at TVCSC, COVAS, Parbhani.

Sr. no.	Sample no.	Breed	Age (in Months)	Sex	Vaccination Status	
1	CPV1	ND	6.0	Male	NV	Parbhani
2	CPV2	German shephard	2.0	Male	NV	Parbhani
3	CPV3	ND	8.0	female	NV	Parbhani
4	CPV4	Labrador	6.0	Male	NV	Parbhani
5	CPV5	ND	2.5	Male	NV	Parbhani
6	CPV6	Pomerian	2.0	Female	V	Parbhani
7	CPV7	ND	7.0	Male	NV	Parbhani
8	CPV8	ND	3.0	Male	NV	Parbhani
9	CPV9	Doberman	4.5	female	V	Hingoli
10	CPV10	ND	8.5	female	NV	Parbhani
11	CPV11	ND	7.0	Male	NV	Parbhani
12	CPV12	ND	1.0	Male	NV	Parbhani
13	CPV13	Labrador	3.0	Female	NV	Parbhani
14	CPV14	ND	9.0	Male	NV	Parbhani
15	CPV15	German shephard	3.0	Male	V	Parbhani
16	CPV16	ND	6.0	Male	NV	Parbhani
17	CPV17	ND	2.5	Male	NV	Parbhani
18	CPV18	Doberman	2.0	female	V	Parbhani
19	CPV19	German shephard	1.8	Male	V	Hingoli
20	CPV20	ND	6.0	Male	NV	Parbhani
21	CPV21	ND	3.0	Male	NV	Parbhani
22	CPV22	ND	5.5	Male	NV	Parbhani
23	CPV23	ND	4.0	Male	NV	Hatta
24	CPV24	Labrador	6.0	Male	NV	Nanded
25	CPV25	ND	4.0	Male	NV	Parbhani

ND – Non Descript V – Vaccinated NV- Non- Vaccinated

Table 3.2 Breed and Sex wise symptoms

No.	Breed	Sex	Symptoms
1	ND	Male	Vomition, diarrhea
2	German shephard	Male	High fever, diarrhea
3	ND	female	Vomition
4	Labrador	Male	Vomition, diarrhea
5	ND	Male	Fever , dehydration
6	Pomerian	Female	diarrhea
7	ND	Male	Vomition, diarrhea
8	ND	Male	diarrhea
9	Doberman	female	Vomition, diarrhea
10	ND	female	diarrhea
11	ND	Male	Bloody diarrhea, dehydration
12	ND	Male	diarrhea
13	Labrador	Female	Vomition, diarrhea
14	ND	Male	diarrhea
15	German shephard	Male	Vomition, bloody diarrhea
16	ND	Male	Diarrhea,
17	ND	Male	Diarrhea ,Dehydration
18	Doberman	female	Vomition, diarrhea
19	German shephard	Male	Fever, diarrhea
20	ND	Male	Weakness
21	ND	Male	Vomition, diarrhea
22	ND	Male	Diarrhea
23	ND	Male	Vomition, bloody diarrhea
24	Labrador	Male	Blood tinched diarrhea
25.	ND	Male	Vomition, diarrhea

3.3 Viral DNA Extraction

3.3.1 Processing of fecal samples

The fecal samples were collected on ice pack. All the fecal samples were diluted to make 10% suspension of each faecal sample using lysis buffer. The diluted faecal sample was centrifuged at 10,000 rpm for 15 minutes for the removal of fine particles and the cellular components. The cleared suspension was stored at -20°C for later use**.**

Table 3.3 Reagents for DNA Isolation

Reagents for DNA Isolation	Uses
Proteinase K 20µg/µl (HiMedia, India)	Proteinase K was dissolved in distilled water and incubated at 37ºC for1 hour, stored at –20ºC for further use
Phenol: Chloroform: Isoamyl alcohol mixture (HiMedia, India)	Phenol: Chloroform: Isoamyl alcohol (25:24:1 v/v/v) mixture was used for extraction of DNA and stored at Refrigeration temperature of 2^0 to 8^0C.
Chloroform	It was kept in a dark amber coloured glass bottle and stored at –20°C and was used for extraction of DNA.
Absolute alcohol **3M Sodium Acetate (pH 5. 5)** **70% Ethanol**	 -Sodium acetate : 24.6 g -Triple distilled water : 100 ml -The pH was adjusted to 5.5 with glacial acetic acid and the volume was made up to 100 ml. - Absolute alcohol : 70 ml - Distilled water : 30 ml
50ml of nucleus free water	Stored at -20^0c for future use.

3.3.2 Extraction of viral DNA

The extraction of viral DNA from fecal samples was carried out by Phenol: chloroform: Iso-amyl alcohol (PCI) as per the method described by Kumar *et al*.,(2011).

3.3.2.A) DNA extraction using PCI

1. Two hundred microliters of the fecal suspension was poured in a 1.5 ml size of micro centrifuge tube. Twenty microliters of proteinase K (Hi Media) was added to it.
2. The mixture was mixed well and incubated at 37^0C for 30 minutes.
3. Equal volume of phenol: chloroform: iso-amyl alcohol (25:24:1) (Hi Media) was added, mixed gently and centrifuged at 8000 rpm for 2 minutes using refrigerated centrifuge.

4. The upper aqueous phase was collected into a new micro centrifuge tube and the above procedure of centrifugation was repeated.
5. The upper aqueous layer was transferred to a new micro centrifuge tube and equal volumes of chloroform was added, mixed gently and centrifuged at 8000 rpm for 2 minutes under refrigerated condition.
6. Sodium acetate (3M) was added to one tenth volume of protein free DNA solution (upper aqueous phase) and two volumes of absolute ethanol was added and kept at -20^{0}C for whole night followed by centrifugation at 13000 rpm for 15 minutes.
7. The DNA pellet was re-suspended in 70% ethanol and centrifuged at 13000 rpm for 15 minutes.
8. The DNA pellet was then dried till no more ethanol left in the tube and suspended in 50 microliters of nuclease free water.

3.4 Polymerase chain reaction

The viral DNA was amplified by PCR assay using VP2 gene specific primers.

3.4.1 The Polymerase chain reaction assay

The PCR assay was standardized in our laboratory using VP2 gene specific primers as per method of Sakulwira *et al.,* (2003). An aliquot of 100 ng of DNA template was added along with mixture containing 10pmol of primer 1 and 10 pmol of primer 2 in a 0.2 ml of thin walled PCR tube. The 2X PCR Master Mix (Himedia Laboratories Pvt. Ltd.) containing Taq DNA polymerase, dNTPs, $MgCl_2$ and PCR buffer at optimal concentration was added to the same mixture. All the chemicals were added in 0.2ml PCR tube on ice and final reaction volume was made upto 50 µl by adding nuclease free water (Table 3.4). The ingredients were mixed and spin for 10 seconds and put in thermal cycler (Biometra, Germany) for 35 cycles of amplification (Table 3.6).

3.4.2 Agarose gel electrophoresis

The PCR amplified products were analyzed on agarose gel electrophoresis (AGE). Agarose gel (1 %) was prepared in 1X TAE buffer by dissolving agarose (1 gm) in 100 ml 1X TAE buffer and heating. The agarose solution was cooled to 50^0 C and Ethidium Bromide was added at 1 µl /20 ml of gel. The solution was poured in gel casting tray fitted with comb to form wells. As soon as the gel got solidified, the comb was taken out. The set gel with casting tray was then submerged in the electrophoresis assembly (GeNei) with sufficient quantity of 1X TAE running buffer above the surface of gel. About 5 µl of PCR product was mixed with 15µl of 6X gel loading dye and loaded in well. One well was loaded with 3µl of 100 bp standard molecular weight DNA ladder (Himedia). PCR product amplified by using DNA of CPV vaccine strain was considered as positive control and Nuclease free water was kept as negative control. Electrophoresis was conducted at 60 V/ 50min of gel till dye reached last third of gel. At the end of electrophoresis, the bands were visualized under UV transilluminator in gel documentation system (Biorad, USA) for the band of desired molecular weight. The sizes of bands separated were calculated by comparing with standard 100 bp molecular weight DNA ladder (Himedia).

Table 3.4: Composition of reaction mixture for PCR

Components	**Quantity**
2X PCR Master mix	12.5 µl
Forward Primer (10 pmol/ µl)	1.0 µl
Reverse Primer (10 pmol/ µl)	1.0 µl
Nuclease free water	9.5 µl
DNA template	1.0 µl
Total	25µl

Table 3.5: Sequence and amplicon size of the oligonucleotide (primers)

Primers	Sequences 5′-3′	Amplicon size
VP2F1	5′-TCC AGC AGC TAT GAG ATC-3′	747bp
VP2R1	5′-GATCTGTTG GTAGCA ATAC-3′	747bp
NSF	GACCGTTACTGACATTCGC	≈2200bp
NSR	CGGCGTCAGAAGGGTT	≈2200bp

Table 3.6: PCR condition for full length amplification of VP2 747 bp and NS1 gene for ≈ 2200 bp product

Steps	Temperature	Time	No. of cycles
Initial denaturation	94^0C	10	1
3-step cycling for 35 cycles			
Denaturation	94^0c	30sec	35
Annealing	58^0c	15sec	35
Extension	72^0c	01min	35
Final extension	72^0c	5min	1
End of the PCR cycling	4^0c	α	

3.5 Sequencing

Ten representative CPV positive PCR products were subjected to sequencing. The sequencing services were hired from Vedant, S No 39/3, H No 1043, Yogi Park Off Mumbai Bangalore Highway, Baner, Pune, Maharashtra-411 045 Web: www.geneombiotechnologies.com. The double pass sequencing reactions of ten PCR products were performed with Big dye terminator V3.1 Cycle sequencing Kit (Cat no-4337455, Make-Applied Biosystems) using a 3500 genetic analyzer.

3.6 Sequence Analysis

The sequences obtained of the field samples were subjected to BLAST analysis with GenBank database sequences using BLASTn algorithm available at NCBI blast (*http://blast.ncbi.nlm.nih.gov/Blast*) as per the method of Kaur *et al,*.(2015) to confirm the presence of the gene specificity to CPV. The nucleotide sequences of VP2 gene fragment of CPV were aligned using default parameters of muscle alignment implemented in MEGA 7.0 software (*http://www.megasoftware. net/*) as per the method of Mittal *et al.,*(2014) with 46 sequences including sequences of Indian and foreign CPV isolates as well as vaccine strain retrieved from GenBank *(http://www.ncbi.nlm.nih. gov/genbank/index.html*). Similarly, the deduced amino acid sequences of VP2 (obtained using ExPASy proteomics) were also aligned using muscle alignment with other related 7 amino acid sequences. Percentage nucleotide / amino acid homology and nucleotide / amino acid differences between sample CPV isolate and other reported sequences were analysed using Sequence Identity and Similarity Tool available at (*http://imed.med. ucm.es/Tools/sias.html*).

3.6.1 Evolutionary distances among CPV isolates

Aligned sequences of partial VP2 genes were used to estimate evolutionary distance among themselves. Best fit model for nucleotide substitution was carried out. Variance estimation was measured by 500 bootstrap replicates. Analyses were conducted using maximum composite likelihood model implemented in MEGA7 program (Kumar *et al*., 2016).

3.6.2 Determination of sequence-specific amino acid patterns

The nucleotide sequences under the study were conceptually translated to corresponding protein sequences. Protein sequences of VP2 gene the 39 CPV isolates were retrieved from the NCBI protein data base (https://www.ncbi.nlm.nih.gov/protein/). All the respective 7 amino acid sequences were aligned using the muscle algorithm (Edgar, 2004). Visualization of alignment was achieved with BioEdit software version 7.2.6.1.

3.6.3 3-Dimensional structure prediction

Consensus sequence of the 7 isolates under study was determined. The same consensus sequence was submitted to The Protein Model Portal available at http://www.proteinmodelportal.org/?pid=modelling interactive. The job returned by RaptorX server integrated in above server was used for visualization of 3 dimensional predictions. Helices, coils and active residues are coloured differently and highlighted (Gholami *et al*.,2015).

3.6.4 B cell linear epitope predictions

B cell epitopes on consensus sequence were determined by two methods; i) Bepipred Linear Epitope Prediction (Jespersen *et al.,*2017) and ii) Parker Hydrophilicity. Prediction tool both with default parameters implemented at IEDB Analysis Resource available at http://tools.iedb.org/main/.

3.7 Phylogenetic Analysis

The Phylogenetic analysis was performed with Bayesian time-scaled phylogenetic method as per the method of (Afreen *et al.,* 2016).

Maximum clade credibility (MCC) phylogenetic tree was constructed by using Bayesian Markov Chain Monte Carlo (MCMC) analysis implemented in Bayesian evolutionary analysis sampling trees (BEAST) software package v2.4.2, and BEAST runs were performed by using CIPRES Science Gateway v.3.3 (https://www.phylo.org/portal2). To perform phylogenetic analysis, the data set of 46 Canine Parvovirus VP2 gene related sequences (07 CPV sequences obtained in the present study and 39 sequences retrieved from NCBI Genbank data base) as shown in (Table 3.7) were aligned using muscle alignment implemented in MEGA7 software (Edgar *et al*., 2004). Branches associated with viruses are shaded by region of origin. In this study 4 groups of the regions were made. In American group, nucleotide sequences from USA and Uruguay are collated together. CPV-2a sequence from Republic of Korea included in Asian group. The Asia group contains CPV sequences from China only. In European group, sequences from Italy. The Indian group included CPV-2 sequences from India

only. The sequences of CPV obtained in the present study were also included in Indian group.

Time to the most recent common ancestor (TMRCA) of CPV-2 strains was assessed using Bayesian inferences implemented in BEASTv2.4.2. Best fit nucleotide substitution model was determined by Akaike Information Criterion (AIC) using jModeltest2 software (Dorriba *et al.,*2012). An input 'xml' file for BEAST analysis was obtained by using Bayesian evolutionary analysis utility software Beauti v2.4.2 in which sequences were dated according to the year of isolation. Both strict and relaxed exponential molecular clocks (Drummond *et al.,* 2006) were used in the analysis. Coalescent constant size and coalescent exponential tree prior were evaluated in the study. The best models were selected by means of Bayes factor (BF) test using marginal likelihood values obtained from Tracer 1.6.0 software. (http://beast.bio.ed.ac.uk/tracer).

Four independent MCMC chains were run. During MCMC, the parameters were visited for 70,000,000 generations and sampled every 7000 cycles resulting in 10,000 trees (ESS >200 for all the parameters estimated) and were assessed for their proper mixing, convergence and consistency by Tracer v.1.6.0 with 10% burn in. The 4 individual runs were combined using LogCombiner in the BEAST software package. The posterior tree distributions were summarized by using Tree Annotator (http://beast.bio.ed.ac.uk/treeannotator) and exclusion of the first 10% of the trees as burn in. Nodes having posterior probabilities higher than 0.9 were included. Phylogenetic tree with mean node heights were visualized in FigTree software v.1.4.2 available at (http:// www.molecularevolution.org /software/ phylogenetics/ fig tree). The uncertainty in the parameter estimates were assessed by 95% HPD (Highest posterior density- posterior distribution of phylogenetic trees is to rank the tree topologies by posterior probability and consider the smallest set of trees that represents at least 95% of the posterior probability) intervals. Bars depict the 95% HPD intervals of the time estimates. The scale on x- axis denotes years.

Table 3.7: Sequences obtained from the GenBank for use in Phylogenetic comparison

Sr. no.	Isolate ID	Year	Place	Gen Bank accession no.
1	CPV1310	2021	Parbhani Maharashtra	
2	CPV1311	2021	Parbhani Maharashtra	
3	CPV1312	2021	Parbhani Maharashtra	
4	CPV1314	2021	Parbhani Maharashtra	
5	CPV1315	2021	Parbhani Maharashtra	
6	CPV1316	2021	Parbhani Maharashtra	
7	CPV1317	2021	Parbhani Maharashtra	
8	CC1-103	2017	China Asia	MN810884.1
9	CPVTS8	2019	China Asia	MT179769.1
10	CN/HN1715	2019	China Asia	MK517980.1
11	QIACPV1404	2014	Republic of Korea Asia	KP893078.1
12	CPV/BJ137	2014	China Asia	KR869671.1
13	CPV canine LZ 1	2017	China Asia	MH155192.1
14	CPV-BJL3	2016	China Asia	MH106700.1
15	JSNT-41	2019	China Asia	MW048561.1
16	GuangZhou Z2	2016	China Asia	KY968643.1
17	IZSSI PA1464/19 idYV8	2018	Nigeria Africa	MK895484.1
18	CC-517	2018	China Asia	MN810888.1
19	CPV/PBN/09/15	2015	India Asia	KX766021.1
20	CPV/PBN/08/15	2015	India Asia	KX766020.1
21	CPV/PBN/02/15	2015	India Asia	KX766014.1
22	CPV/PBN/03/15	2015	India Asia	KX766015.1
23	China/22	2017	China Asia	MH476591.1
24	guangzhou/GZ-4	2017	China Asia	KY937660.1
25	GY-6	2016	China Asia	KY386855.1
26	18Q26-1-1	2018	South Korea Asia	MN453223.1
27	CH-AH-D14	2019	China Asia	MN119573.1

28	20160810-BJ-19	2016	China Asia	MF347728.1
29	20170322-BJ-25	2017	China Asia	MF347738.1
30	XuZhou/01g/2016	2016	China Asia	KY922908.1
31	18Q124-2	2018	South Korea Asia	MN053898.1
32	CPV-HN1618	2016	China Asia	MF467228.1
33	G5 2009	2009	China Asia	KF482469.1
34	CPV/PBN/07/15	2015	India Asia	KX766019.1
35	CPV/PBN/05/15	2015	India Asia	KX766017.1
36	CPV/PBN/01/15	2015	India Asia	KX766013.1
37	2009	2009	India Asia	KC713932.1
38	GA/11/11	2011	USA America	JX475238.1
39	M169 2008	2008	Uruguay America	KC196104.1
40	217/07 2008	2008	Italy Europe	FJ005242.1
41	M129 2008	2008	Uruguay America	KC196107.1
42	M173 2009	2009	Uruguay America	KC196103.1
43	Arg60 2009	2009	Argentina America	JF414823.1
44	M152 2008	2008	Uruguay America	KC196105.1
45	08/09 2009	2009	China Asia	GU380305.1
46	ME32 ECU2012 VP2	2012	Uruguay America	KF149971.1

Results and Discussion

CHAPTER - IV

RESULTS AND DISCUSSION

The canine parvovirus is a highly contagious disease of dogs of all ages but, in pups the severity is higher. Dogs get exposed to the parvovirus while sniffing, licking, or consumption of CPV contaminated object. In India there were several outbreaks of canine parvovirus mainly in non-vaccinated dogs but reports of CPV infection in vaccinated dogs have raised the concerns for detection of strain variation of CPV. The present study was conducted in order to find out the CPV strain/s prevalent in Marathawada region among the dogs infected with CPV.

4.1 Collection samples and management

The fecal samples (n=25) were collected from dogs that were suffered with diarrhea from various areas of Parbhani and Teaching Veterinary Clinical Complex (TVCC), Parbhani (Plate 4.1). The samples were marked with species, sex, serial number, date and place of collection. The dogs exhibited various clinical signs such as vomition and diarrhea. In pups symptoms observed were high body temperature (105.1^0F), inapatence, inability to walk and weakness. In few dogs there was history of vomition and bloody diarrhea for long time. In our study vaccinated dogs were also observed to exhibit vomition, diarrhea and high fever. In case of non-descript dogs it was observed that dogs were emaciated and severely dehydrated. The recovery of healthy dogs was faster as compared to weak dogs.

Decaro *et al*., (2012) in his study on CPV infected dogs reported clinical signs of anorexia, depression, vomiting and mucoid or bloody diarrhea, frequently dehydration and fever which were similar to the clinical signs observed in the present study. Mylonakis *et al*.,(2016) also observed clinical signs of anorexia, lethargy, weakness, depression, foul-smelling diarrhea varied from mucoid to purely haemorrhagic stools, vomiting, dehydration and fever having similarity with our clinical signs observed in some of the dogs affected with CPV.

In the present study no mortality was recorded among the dogs under study. The infected dogs were recovered within 2-3 weeks.

4.2 DNA Extraction:

In the present study DNA was extracted from the fecal samples (n=25) of dogs by phenol: Chloroform: isoamyl method (PCI) for the amplification of VP2 specific gene primers. We preferred the PCI method over boiling and kit method as the quality of DNA was good as compared to boiling method and was cheaper as compared to kit method. Desai *et al*., (2020) also carried out DNA extraction by PCI method of CPV fecal sample and they reported that PCI method was time consuming method than boiling and kit method. Kok *et al*., (2000) stated that average concentration of DNA yield was high in PCI method which is in agreement with our study.

4.3 Molecular identification by Polymerase chain reaction

Polymerase chain reaction (PCR) is a confirmatory laboratory test that amplifies the target DNA sequences using short oligonucleotide sequences called primers to select the portion of the gene/ genome to be amplified. In this study we characterized CPV in Marathawada region of Maharashtra state by PCR using VP2 gene specific primers which is a major envelope protein with strong immunogenic potential commonly observed in CPV.

The amplified PCR products after 35 cycles were subjected to agar gel electrophoresis on 1 % agarose gel for visualization of bands of specific VP2 gene that revealed amplicon product size of 747 bp, as determined by comparison with 100 bp DNA ladder. In our study, out of 25 DNA's extracted from fecal samples, 23 (92%) were found to be positive as it revealed a single and uniform band of 747 bp (plate 4.2) size on 1% agarose gel and 2 samples did not exhibit any band by PCR were considered to be negative for CPV. The bandwidth of 747 bp was comparable with that of PCR product of CPV vaccine strain which was kept as positive control. Negative control (N) of nuclease free water did not show any band due to non-amplification and absence of CPV. These observations lead to the conclusion that the 23 samples were positive for CPV by PCR. In a similar

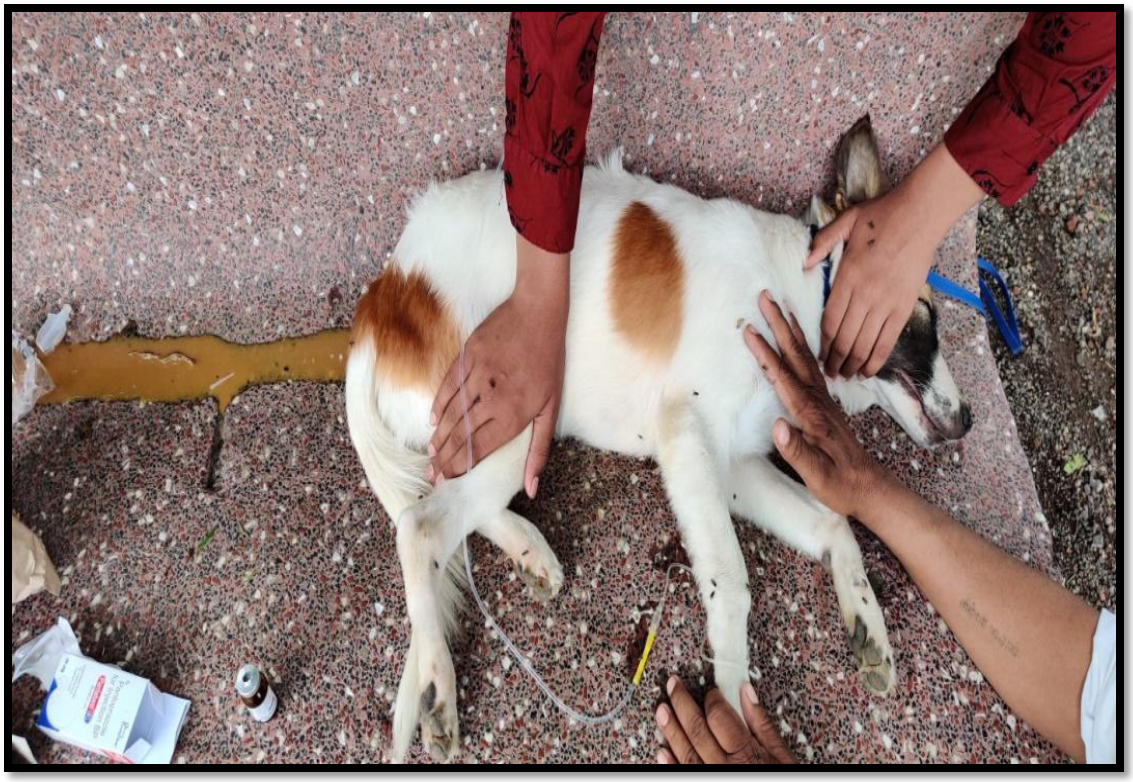


Fig.4.1 Photographs of CPV infected Dogs

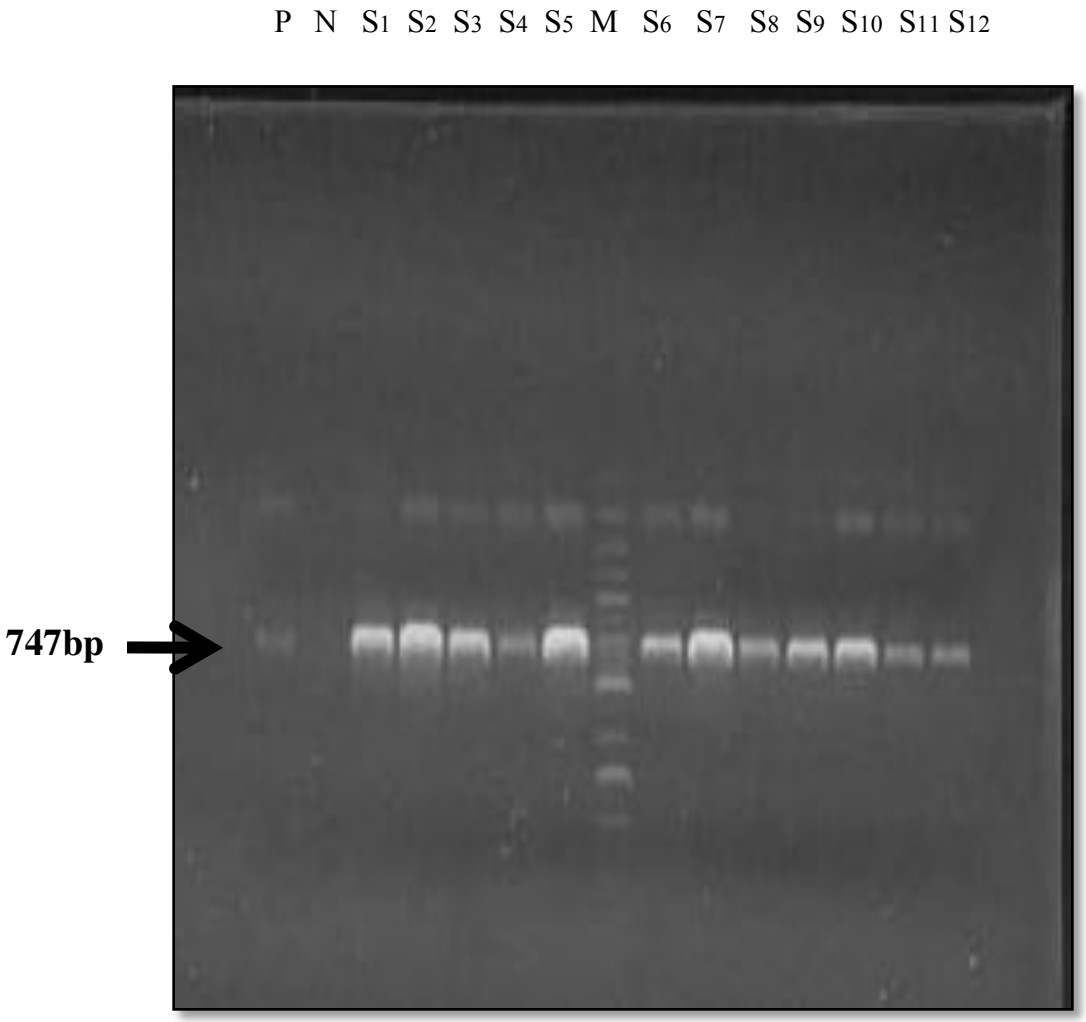


Plate 1: **Polymerase chain reaction (PCR) used for determination of canine parvovirus (CPV) with amplicon size 747 bp of VP2 gene.**

M **= 100 bp marker**

P **= PCR positive control**

N **= Negative control**

S1-S12 **= PCR samples 1-12**

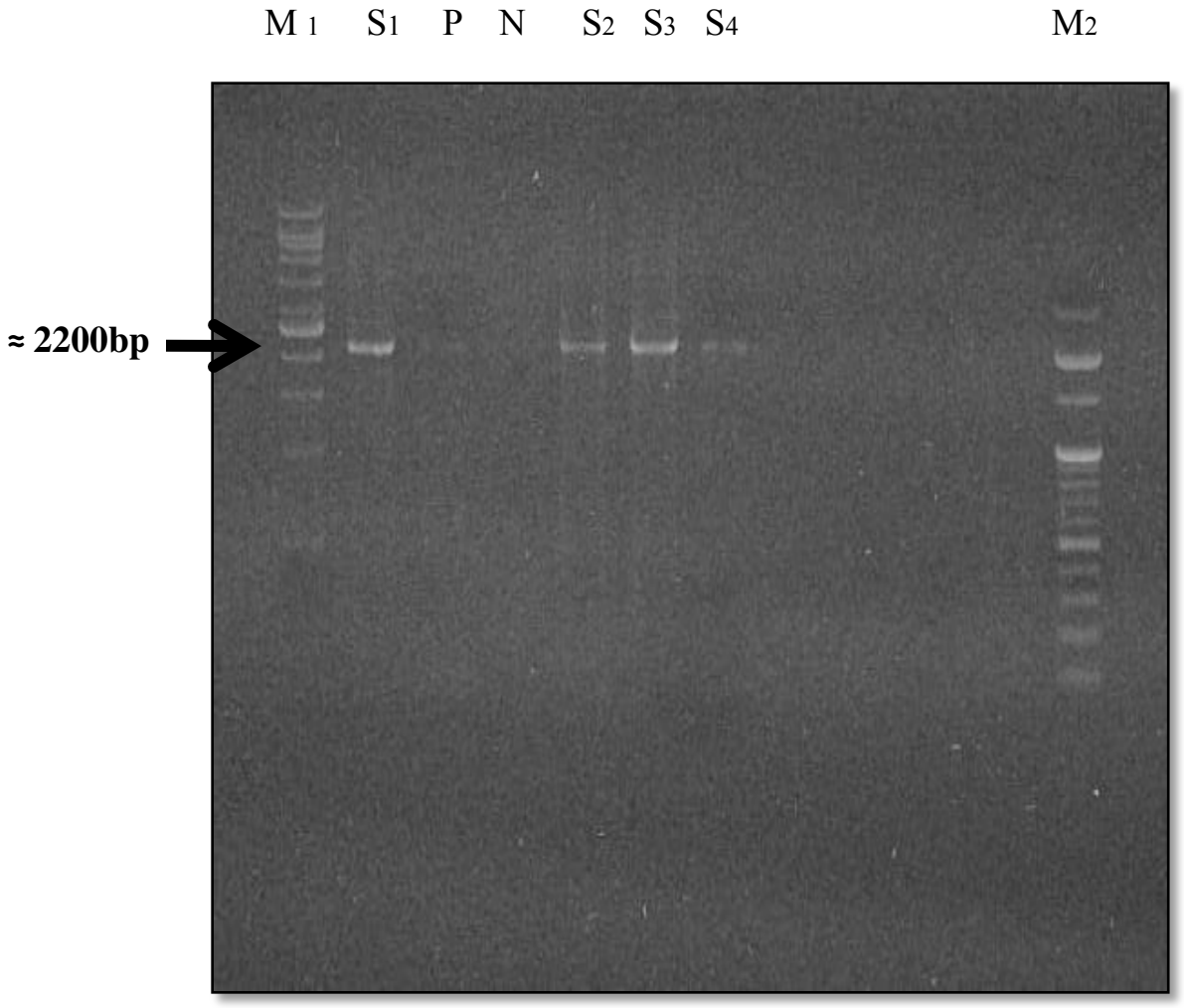


Plate 2: **Polymerase chain reaction (PCR) used for determination of canine parvovirus (CPV) with amplicon size ≈ 2200 bp of NS 1 gene.**

M 1 **= 100 bp marker**

M 2 **= 1 kb marker**

P **= PCR positive control**

N **= Negative control**

S1-S4 **= PCR samples 1-4**

study by kulkarni *et al*., (2019) who performed PCR by VP2 gene specific primers and got amplified product of single and uniform band 747 bp size of 9 fecal samples that were confirmed to be positive for CPV infection. Chinchkar *et al*., (2006) also carried out PCR using primers specific for CPV VP2 gene type 2a/2b and got an amplicon with a of size 680 bp, indicative of the variants CPV type 2a or 2b.Singh *et al*.,(2021) processed for DNA isolation and polymerase chain reaction (PCR) for VP2 gene at 1198 bp and nested PCR (NPCR) at 548 bp and reported the percent positive of CPV was determined to be 28% and 70%, respectively.

In addition to VP2 specific gene primers for identification we used NS1gene primer with the amplicon size ≈ 2200 bp. A total of 14 fecal samples DNA were tested for PCR amplification. It was observed that only 5 samples exhibited ≈ 2200 bp amplified product size were considered to be positive for CPV (plate 4.3). Mokhtari *et al*., (2018) in a similar study detected CPV using CPV-NS1 nucleotide fragment PCR amplification to obtain CPV-specific 150 bp product size in positive samples. They detected 5 samples positive out of 60 samples tested.

4.4 Prevalence

4.4.1 On the basis of Sex

Canine parvovirus VP2 gene PCR amplification showed that a total of 23 samples (92%) were positive for CPV. Out of all samples, a total of 24% (n=6) females fecal samples and 76% (n=19) male faecal samples were confirmed to be positive for CPV. Giraldo-Ramirez *et al*., (2020) studied and observed that a total of 29 samples 51.8% were CPV-2-positive out of that 41.4% (n=12) belonged to females and 58.6% (n=17) were of male dogs which is in agreement to our study.

4.4.2 On the basis of Breed

In the present study CPV was observed in 36% of pure breed dogs and 64% in Non-descript (ND) dogs. Headley *et al*., (2018) showed in their study that there was no difference in the gender (females, 7; males, 8) of the puppies whereas the Pure breed dogs 73.3% were predominantly affected with CPV relative to their mixed breed counterparts 26.7% which was contrary to the observations of our study where we observed more prevalence in ND and mixed breeds. The reason may be that most of the pure breed dogs were vaccinated as compared to ND dogs.

4.4.3 On the basis of Vaccination status.

In this study we observed that CPV infection occurrence was less in vaccinated 5% as compared to unvaccinated 80% dogs. Hasib *et al*., (2021) recorded in their study that vaccination showed no significant association with infection in multivariable analysis, but invariable analysis indicated that the disease occurrence was higher 33% in unvaccinated dogs compared with vaccinated dogs 12% which was agreement with our study. The two dogs that were vaccinated and were confirmed CPV positive may be due to immunisation failure that can be vaccine related and/or host related. Two vaccinated dogs that were found positive were of Doberman breed and it is reported earlier by Houstan *et al*., (1996) that some canine breeds like Doberman and Routviller have been reported to be of higher risk of immunisation failure to CPV vaccines which might be the reason of CPV infection in our study to vaccinated dogs were of Doberman breed. There are various reason of vaccine related failures include vaccine storage non-compliance with vaccine schedules of failure in vaccine immunogenicity (Altman *et al.,*2017) which might be the reasons of CPV positive cases of other vaccinated dogs in this study.

4.5 Gene Sequencing Analysis:

In the present study a total of 23 faecal samples were tested positive for CPV by PCR and 07 representative CPV positive PCR products selected and were sequenced and data were analysed. Sequence information was obtained for a

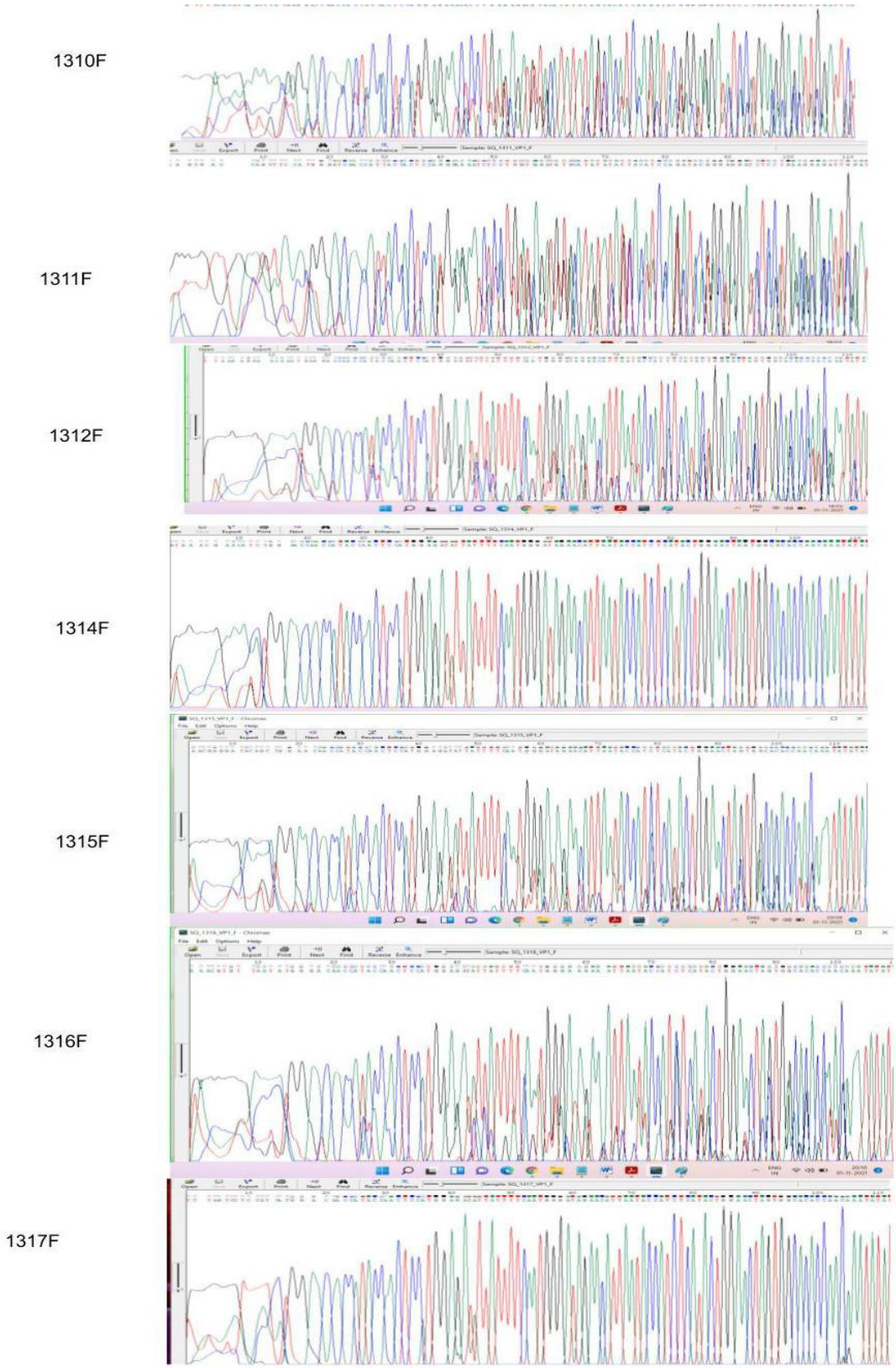


Fig 4.2. Sequence chromatogram of selected region of sequenced PCR sample No. 1 to Sample no. 7

partial sequence of 747 bp long region in VP2 coding sequence. Sequencing results and deduced amino acids are presented in (Table 4.1). Sequencing chromatogram of selected region of sequenced PCR products (sample no.1 to sample no.7) are presented in (plate 4.4). The VP2 gene sequences obtained in the present study were compared with each other and also with other CPV and related viruses VP2 gene sequences available in the GenBank using muscle Alignment implemented in software MEGA 7.0 (Table 4.1). The deduced amino acid sequences were compared between isolates and also with the isolates reported from all over the world. From the sequencing analysis of the deduced amino acids and nucleotides all the 7 CPV sequences were belonged to CPV 2a virus the prevalence dominance of CPV2a was also described by Kulkarni *et al*., (2019). In a similar study Fagbohun *et al*., (2018) sequenced CPV positive DNA of the partial VP2 gene of the seven Nigerian CPV sequences compared them with other published CPV-2 sequences and observed that they belonged to CPV 2a and CPV 2c Sharma *et al*., (2018) also sequenced the CPV virus and observed that the sequences belonged to the CPV 2b type which was not observed in our study the prevalence dominance was observed at Gujrat contrary to our study.

Table 4.1 Accession numbers of nucleotide sequences of CEV submitted to NCBI

Sr no.	Sequence ID	Size of sequence fragments	Year of Sequencing	Place	Gene Bank accession no.
1		747bp	2021	Parbhani Maharashtra	
2		747bp	2021	Parbhani Maharashtra	
3		747bp	2021	Parbhani Maharashtra	
4		747bp	2021	Parbhani Maharashtra	
5		747bp	2021	Parbhani Maharashtra	

Table. 4.2 Nucleotide and deduced amino acid sequences of the CEV

Sr. no	Nucleotide sequence /protein Sequence	Sequence
CPV1310	Nucleotide sequence	GAGATCTGAGACATTGGGTTTTTATCCA TGGAAACCAACCATACCAACTCCATGG AGATATTATTTTCAATGGGATAGAACAT TAATACCATCTCATACTGGAACTAGTGG CACACCAACAAATATATACCATGGTAC AGATCCAGATGATGTTCAATTTTACACT ATTGAAAATTCTGTGCCAGTACACTTAC TAAGAACAGGTGATGAATTTGCTACAG GAACATTTTATTTTGATTGTAAACCATG TAGACTAACACATACATGGCAAACAAA TAGAGCATTGGGCTTACCACCATTTCTA AATTCTTTGCCTCAAGCTGAAGGAGGTA CTAACTTTGGTTATATAGGAGTTCAACA AGATAAAAGACGTGGTGTAACTCAAAT GGGAAACACAAACATTATTACTGAAGC TACTATTATGAGACCAGCTGAGGTTGGT TATAGTGCACCATATTATTCTTTTGAGG CGTCTACACAAGGGCCATTTAAAACAC CTATTGCAGCAGGACGGGGGGGAGCGC AAACAGATGAAAATCGAGCAGCAGATG GTGATCCAAGATATGCATTTGGTAGAC AACATGGTCAAAAAACTACCACAACAG GAGAAACACCTGAGAGATTTACATATA TAGCACATCAAGATACAGGAAGATATC CAGAAGGAGATTGGATTCAAAATATTA ACTTTAACCTTCCTGTAACAGAAGATAA TGTAT
	Protein Sequences	RSETLGFYPWKPTIPTPWRYYFQWDRTLI PSHTGTSGTPTNIYHGTDPDDVQFYTIENS VPVHLLRTGDEFATGTFYFDCKPCRLTHT WQTNRALGLPPFLNSLPQAEGGTNFGYIG VQQDKRRGVTQMGNTNIITEATIMRPAE VGYSAPYYSFEASTQGPFKTPIAAGRGGA QTDENRAADGDPRYAFGRQHGQKTTTTG ETPERFTYIAHQDTGRYPEGDWIQNINFN LPVTEDNV
CPV1311	Nucleotide sequence	GCTATGAGATCTGAGACATTGGGTTTTT ATCCATGGAAACCAACCATACCAACTC CATGGAGATATTATTTTCAATGGGATAG AACATTAATACCATCTCATACTGGAACT AGTGGCACACCAACAAATATATACCAT GGTACAGATCCAGATGAAGTTCAAGAT TATAGTATTGAACATTCAGAGCCAGTAC ACTTACTAAGAACAGGTGATGAATTTG

		CTACAGGAACATTTTATTTTGATTGTAA ACCATGTAGACTAACACATACATGGCA AACAAATAGAGCATTGGGCTTACCACC ATTTCTAAATTCTTTGCCTCAAGCTGAA GGAGGTACTAACTTTGGTTATATAGGA GTTCAACAAGATAAAAGACGTGGTGTA ACTCAAATGGGAAACACAAACATTATT ACTGAAGCTACTATTATGAGACCAGCT GAGGTTGGTTATAGTGCACCATATTATT CTTTTGAGGCGTCTACACAAGGGCCATT TAAAACACCTATTGCAGCAGGACGGGG GGGAGCGCAAACAGATGAAAATCGAGC AGCAGATGGTGATCCAAGATATGCATT TGGTAGACAACATGGTCAAAAAACTAC CACAACAGGAGAAACACCTGAGAGATT TACATATATAGCACATCAAGATACAGG AAGATATCCAGAAGGAGATTGGATTCA AAATATTAACTTTAACCTTCCTGTAACA GAAGATAATGTAT
	Protein Sequence	AMRSETLGFYPWKPTIPTPWRYYFQWDR TLIPSHTGTSGTPTNIYHGTDPDEVQDYSI EHSEPVHLLRTGDEFATGTFYFDCKPCRL THTWQTNRALGLPPFLNSLPQAEGGTNF GYIGVQQDKRRGVTQMGNTNIITEATIMR PAEVGYSAPYYSFEASTQGPFKTPIAAGR GGAQTDENRAADGDPRYAFGRQHGQKT TTTGETPERFTYIAHQDTGRYPEGDWIQN INFNLPVTEDNV
CPV1312	Nucleotide sequence	TGAGATCTGAGACATTGGGTTTTTATCC ATGGAAACCAACCATACCAACTCCATG GAGATCTTATTTTCAATGGGATAGAACA TTAATACCATCTCATACTGGAACTAGTG GCACACCAACAAATATATACCATGGTA CAGATCCTGATGATGTTCAATTTTACAC TATGGAAAATTCTGTGCAAGTACACTTA CTAAGAACAGGTGATGAATTTGCTACA GGAACATTTTATTTTGATTGTAAACCAT GTAGACTAACACACACATGGCAAACAA ATAGAGCATTGGGCTTACCACCATTTCT AAATTCTTTGCCTCAAGCTGAAGGAGGT ACTAACTTTGGTTATATAGGAGTTCAAG AAGATAAAAGACGTGGTGTAACTCAAA TGGGAAATACAAACATTATTACTGAAG CTACTATTATGAGACCAGCTGAGGTTGG TTATAGTGCACCATATTATTCTTTTGAG GCGTCTACACAAGGGCCATTTAAAACA CCTATTGCAGCAGGACGGGGGGGAGCG CAAACAGATGAAAATCAAGCAGCAGAT

		GGTGATCCAAGATATGCATTTGGTAGA CAACATGGTCAAAAAACTACCACAACA GGAGAAACACCTGAGAGATTTACATAT ATAGCACATCAAGATACAGGAAGATAT CCAGAAGGAGATTGGATTCAAAATATT AACTTTAACCTTCCTGTAACAAATGATA ATGTAT
	Protein sequence	RSETLGFYPWKPTIPTPWRSYFQWDRTLI PSHTGTSGTPTNIYHGTDPDDVQFYTMEN SVQVHLLRTGDEFATGTFYFDCKPCRLTH TWQTNRALGLPPFLNSLPQAEGGTNFGYI GVQEDKRRGVTQMGNTNIITEATIMRPAE VGYSAPYYSFEASTQGPFKTPIAAGRGGA QTDENQAADGDPRYAFGRQHGQKTTTT GETPERFTYIAHQDTGRYPEGDWIQNINF NLPVTNDNV
CPV1314	Nucleotide sequence	TGAGATCTGAGACATTGGGTTTTTATCC ATGGAAACCAACCATACCAACTCCATG GAGATATTATTTTCAATGGGATAGAAC ATTAATACCATCTCATACTGGAACTAGT GGCACACCAACAAATATATACCATGGT ACAGATCCAGATGATGTTCAATTTTACA CTATTGAAAATTCTGTGCCAGTACACTT ACTAAGAACAGGTGATGAATTTGCTAC AGGAACATTTTTTTTGATTGTAAACCA TGTAGACTAACACACACATGGCAAACA AATAGAGCATTGGGCTTACCACCATTTC TAAATTCTTTGCCTCAAGCTGAAGGGGG TACTAACTTTGGTTATATAGGAGTTCAA CAAGATAAAAGACGTGGTGTAACTCAA ATGGGAAATACAAACATTATTACTGAA GCTACTATTATGAGACCAGCTGAGGTTG GTTATAGTGCACCATATTATTCTTTTGA GGCGTCTACACAAGGGCCATTTAAAAC ACCTATTGCAGCAGGACGGGGGGGAGC GCAAACAGATGAAAATCAAGCAGCAGA TGGTGATCCAAGATATGCATTTGGTAGA CAACATGGTCAAAAAACTACCACAACA GGAGAAACACCTGAGAGATTTACATAT ATAGCACATCAAGATACGGGAAGATAT CCAGAAGGAGATTGGATTCAAAATATT AACTTTAACCTTCCTGTAACAGATGATA ATGTAT
	Protein Sequence	RSETLGFYPWKPTIPTPWRYYFQWDRTLI PSHTGTSGTPTNIYHGTDPDDVQFYTIENS VPVHLLRTGDEFATGTFFFDCKPCRLTHT WQTNRALGLPPFLNSLPQAEGGTNFGYIG VQQDKRRGVTQMGNTNIITEATIMRPAE

		VGYSAPYYSFEASTQGPFKTPIAAGRGGA QTDENQAADGDPRYAFGRQHGQKTTTT GETPERFTYIAHQDTGRYPEGDWIQNINF NLPVTDDNV
CPV1315	Nucleotide Sequence	CTGAGACATTGGGTTTTTATCCATGGAA ACCAACCATACCAACTCCATGGAGATA TTATTTTCAATGGGATAGAACATTAATA CCATCTCATACTGGAACTAGTGGCACAC CAACAAATATATACCATGGTACAGATC CTGATGATGTTCAATTTTACACTATTGA AAATTCTGTGCAAGTACACTTACTAAGA ACAGGTGATGAATTTGCTACAGGAACA TTTTATTTTGATTGTAAACCATGTAGAC TAACACACACATGGCAAACAAATAGAG CATTGGGCTTACCACCATTTCTAAATTC TTTGCCTCAAGCTGAAGGGGGTACTAA CTTTGGTTATATAGGAGTTCAACAAGAT AAAAGACGTGGTGTAACTCAAATGGGA AATACAAACATTATTACTGAAGCTACTA TTATGAGACCAGCTGAGGTTGGTTATAG TGCACCATATTATTCTTTTGAGGCGTCT ACACAAGGGCCATTTAAAACACCTATT GCAGCAGGACGGGGGGGAGCGCAAAC AGATGAAAATCAAGCAGCAGATGGTGA TCCAAGATATGCATTTGGTAGACAACAT GGTCAAAAAACTACCACAACAGGAGAA ACACCTGAGAGATTTACATATATAGCA CATCAAGATACGGGAAGATATCCAGAA GGAGATTGGATTCAAAATATTAACTTTA ACCTTCCTGTAACAGATGATAATG
	Protein Sequence	ETLGFYPWKPTIPTPWRYYFQWDRTLIPS HTGTSGTPTNIYHGTDPDDVQFYTIENSV QVHLLRTGDEFATGTFYFDCKPCRLTHT WQTNRALGLPPFLNSLPQAEGGTNFGYIG VQQDKRRGVTQMGNTNIITEATIMRPAE VGYSAPYYSFEASTQGPFKTPIAAGRGGA QTDENQAADGDPRYAFGRQHGQKTTTT GETPERFTYIAHQDTGRYPEGDWIQNINF NLPVTDDN
CPV1316	Nucleotide Sequence	TGAGACATTGGGTTTTTATCCATGGAAA CCAACCATACCAACTCCATGGAGATATT ATTTTCAATGGGATAGAACATTAATACC ATCTCATACTGGAACTAGTGGCACACC AACAAATATATACCATGGTACAGATCC TGATGATGTTCAATTTTACACTATTGAA AATTCTGTGCAAGTACACTTACTAAGAA CAGGTGATGAATTTGCTACAGGAACAT TTTTTTTTGATTGTAAACCATGTAGACT

		AACACACACATGGCAAACAAATAGAGC ATTGGGCTTACCACCATTTCTAAATTCT TTGCCTCAAGCTGAAGGGGGTACTAAC TTTGGTTATATAGGAGTTCAACAAGATA AAAGACGTGGTGTAACTCAAATGGGAA ATACAAACATTATTACTGAAGCTACTAT TATGAGACCAGCTGAGGTTGGTTATAGT GCACCATATTATTCTTTTGAGGCGTCTA CACAAGGGCCATTTAAAACACCTATTG CAGCAGGACGGGGGGGAGCGCAAACA GATGAAAATCAAGCAGCAAATGGTGAT CCAAGATATGCATTTGGTAGACAACAT GGTCAAAAAACTACCACAACAGGAGAA ACACCTGAGAGATTTACATATATAGCA CATCAAGATACGGGAAGATATCCAGAA GGAGATTGGATTCAAAATATTAACTTTA ACCTTCCTGTAACAGATGATAATGTAA
	Protein Sequence	ETLGFYPWKPTIPTPWRYYFQWDRTLIPS HTGTSGTPTNIYHGTDPDDVQFYTIENSV QVHLLRTGDEFATGTFFFDCKPCRLTHT WQTNRALGLPPFLNSLPQAEGGTNFGYIG VQQDKRRGVTQMGNTNIITEATIMRPAE VGYSAPYYSFEASTQGPFKTPIAAGRGGA QTDENQAANGDPRYAFGRQHGQKTTTT GETPERFTYIAHQDTGRYPEGDWIQNINF NLPVTDDNV
CPV1317	Nucleotide Sequence	TGAGATCTGAGACATTGGGTTTTTATCC ATGGAAACCAACCATACCAACTCCATG GAGATATTATTTTCAATGGGATAGAAC ATTAATACCATCTCATACTGGAACTAGT GGCACACCAACAAATATATACCATGGT ACAGATCCAGATGATGTTCAATTTTACA CTATTGAAAATTCTGTGCCAGTACACTT ACTAAGAACAGGTGATGAGTTTGCTAC AGGAACATTTTATTTTGATTGTAAACCA TGTAGACTAACACACACATGGCAAACA AATAGAGCATTGGGCTTACCACCATTTC TAAATTCTTTGCCTCAAGCTGAAGGAGG TACTAACTTTGGTTATATAGGAGTTCAA GAAGATAAAAGACGTGGTGTAACTCAA ATGGGAAATACAAACATTATTACTGAA GCTACTATTATGAGACCAGCTGAGGTTG GTTATAGTGCACCATATTATTCTTTTGA GGCGTCTACACAAGGGCCATTTAAAAC ACCTATTGCAGCAGGACGGGGGGGAGC GCAAACAGATGAAAATCAAGCAGCAGA TGGTGATCCAAGATATGCATTTGGTAGA CAACATGGTCAAAAAACTACCACAACA

		GGAGAAACACCTGAGAGATTTACATAT ATAGCACATCAAGATACAGGAAGATAT CCAGAAGGAGATTGGATTCAAAATATT AACTTTAACCTTCCTGTAACAAATGATA ATGTAT
	Protein sequence	RSETLGFYPWKPTIPTPWRYYFQWDRTLI PSHTGTSGTPTNIYHGTDPDDVQFYTIENS VPVHLLRTGDEFATGTFYFDCKPCRLTHT WQTNRALGLPPFLNSLPQAEGGTNFGYIG VQEDKRRGVTQMGNTNIITEATIMRPAEV GYSAPYYSFEASTQGPFKTPIAAGRGGAQ TDENQAADGDPRYAFGRQHGQKTTTTGE TPERFTYIAHQDTGRYPEGDWIQNINFNL PVTNDNV

Table. 4.3 Amino acid variation in the VP2 capsid protein of the Parbhani CPV isolates

		Amino Acids Variation														
		Position														
Strain	Year	Acc.no	122	210	240	243	245	246	248	250	251	267	310	370	373	426
2a	2021	PBN1310	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	E	ND	ND	E
2a	2021	PBN1311	ND	ND	E	D	S	ND	H	E	ND	ND	ND	R	ND	E
2a	2021	PBN1312	ND	S	ND	ND	ND	M	ND	ND	Q	F	E	ND	ND	ND
2a	2021	PBN1314	ND	ND	ND	ND	ND	ND	ND	ND	ND	F	ND	ND	ND	ND
2a	2021	PBN1315	ND	ND	ND	ND	ND	ND	ND	ND	Q	ND	ND	ND	ND	ND
2a	2021	PBN1316	ND	ND	ND	ND	ND	ND	ND	ND	Q	ND	ND	ND	N	ND
2a	2021	PBN1317	E	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2a		Alc79697	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	N

There were amino acid variations detected in our samples after the sequence analysis of the 7 CPV sequences of the Marathwada region of Maharashtra. Complete VP2 nucleotide sequences obtained from the examined samples were translated into amino acid sequences. Based on analysis of residues of 122, 210, 240, 243, 245, 246, 248, 250, 251, 367, 310, 370, 373, and 426 changes observed which are depicted in (Table 4.3). The changes in nucleotides resulted into corresponding amino acid changes as E→Q (glu310gln), E→N (glu426Asn), E→D (glu240asp), D→Y (asp243Tyr), S→T (ser245Thr), H→N(his248Asn), E→V (glu250 Val), R→Q (arg370gln), E→N (glu426Asn), S→Y (ser210Tyr), M→I (met246IIe), Q→P (gln251phe), F→Y(phe267Tyr), E→Q (glu310gln), F→Y(phe267Tyr), Q→P (gln251phe), Q→P (gln251phe), N→D (Asn373asp). In the sample of PBN1311 there were 7 changes observed at the position of (240, 243, 245, 248, 250, 370, 426), whereas in sample no's PBN1312, PBN1315 and PBN1316 glutamine was observed instead of proline at a same position 251.On the basis of sequences analysis all the samples of our study belonged to CPV 2a strain. Dogonyaro *et al*., (2013) analysed amino acid sequence and observed seven predicted amino acid changes: T→K (Thr265Lys), A→N (Ala297Asn), Y→I (Tyr324IIe), V→A (Val424Ala), N→D (Asn426Asp), T→A (Thr440Ala) and D→N (Asp475Asn) at their various positions with corresponding identity numbers. In their study reported the predicted antigenic amino acids of CPV-2a and 2b, with no report on CPV-2 and 2c. Inthong *et al*.,(2020) detected most of the CPV had amino acid substitution at positions 324 and 440. Eighty-three CPV had 324 (Tyr-Ile) substitutions due to a T-to-A transversion at nucleotide 970 and an A-to-T transversion at nucleotide 971 with the exception of 1 sample of new CPV-2b. Miranda *et al*.,(2016) detected antigenic differences among the three variants were associated with changes at residue 426 Asn in CPV-2A, Asp in CPV-2b and Glu in CPV-2C. But in our study no antigenic changes were observed at 426 position.

In this study residue 373 changed from aspartic acid to asparagine (asp373asn) but there was no change observed in the strain CPV 2a. Miranda *et al*.,(2016) detected residue 375 variation only in few isolates of the original strain of CPV-2, and in later CPV variants that residue reverted to an Asp, suggesting

that 375Asn is not critical to the success of CPV in nature. Nandi *et al*.,(2010) found that substitutions at positions 87 (Met to Leu), 300 (Gly to Ala), 305 (Tyr to Asp) and 555 (Val to Ile) occurred in the evolution of 2 to 2a and 426 (Asn to Asp) and 555 (Ile to Val) that lead to the emergence of CPV 2b from 2a. There were amino acid changes in the capsid protein (VP2), which characterized the shift from 2 to 2a and to 2b but in our study there was no shifting of 2a to 2b. All the sequences of this study were CPV 2a type only. Sharma *et al*.,(2018) reported the sequences of CPV-2b with 98–99% identity matched with submitted sequences. When those were aligned with submitted Indian or foreign sequences, some major nucleotide differences were found at a different position, particularly with CPV-2a types which were not observed in our study.

4.6 3D model of Protein Variation

The three-dimensional atomic structure of a VP2 partial protein sequence model after template VP2 sequence has been determined. The amino acid sequences were variable as per (table no 4.2) the variations of amino acids are highlighted in red colour. The 3D model of CPV indicates protein variation sites in the VP2 gene structure. The CPV 2a strain is indicated in red colour on the surface of model (plate 4.5).

4.7 B cell linear epitope predictions:

The two epitopes predicted by algorithm epitopes are 79-NVTLLVDVQSKDKDADE-95 and 300-SVDMSVRKFVV-310. Both epitopes fall within regions identified as having high levels of antigenicity. The two-dimensional protein structures predicted X B cell epitopes in the six and nine epitopes in the sequenced region respectively with antigenicity with scores between 0.5 and 0.7 by Bepipred Method and 2 and 6 by Parker Method (plate 4.6). In similar study Sharma *et al.,* (2020) have used the Bepipred method for B cell linear epitope predictions for canine parvovirus. B-cell epitopes lies in between the 59-75 amino acid positions.

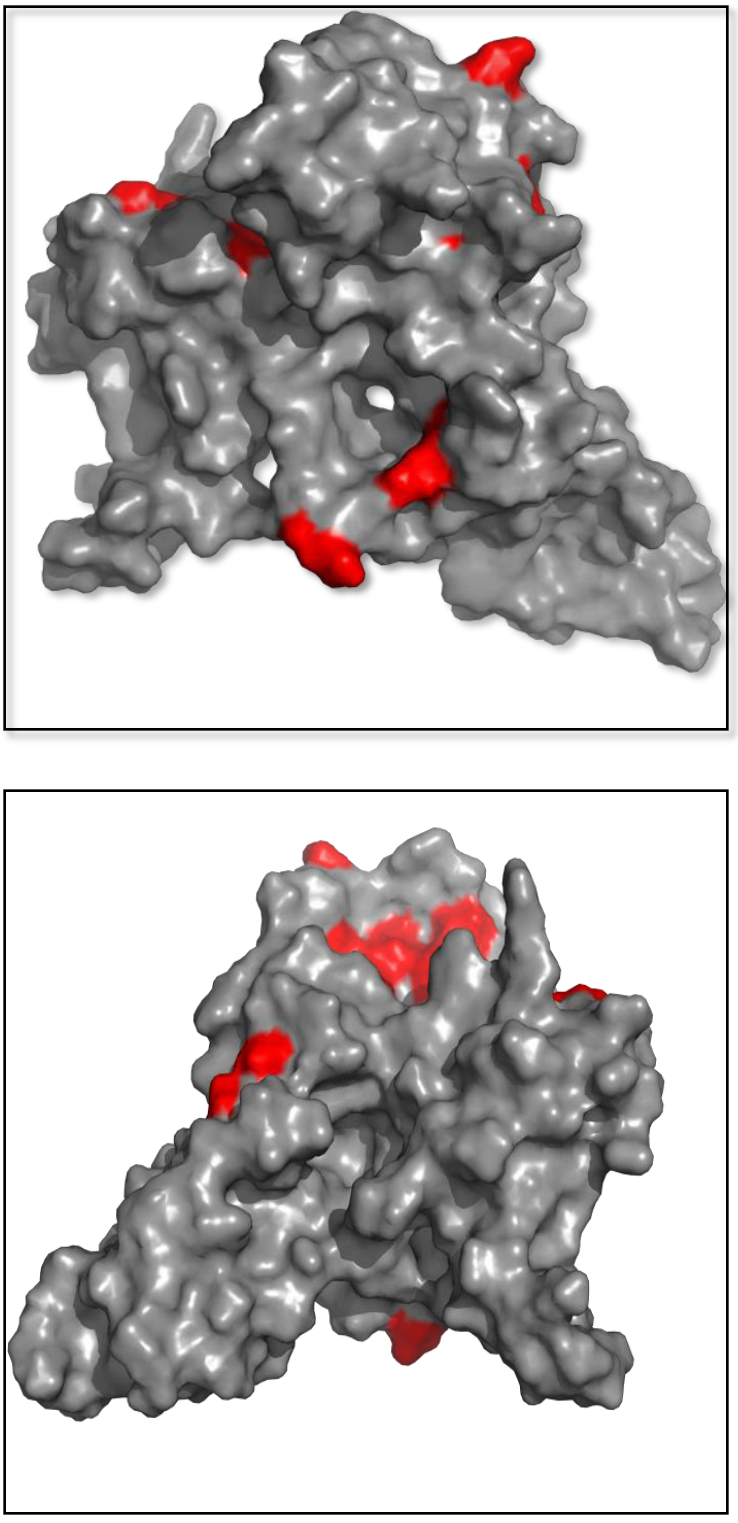
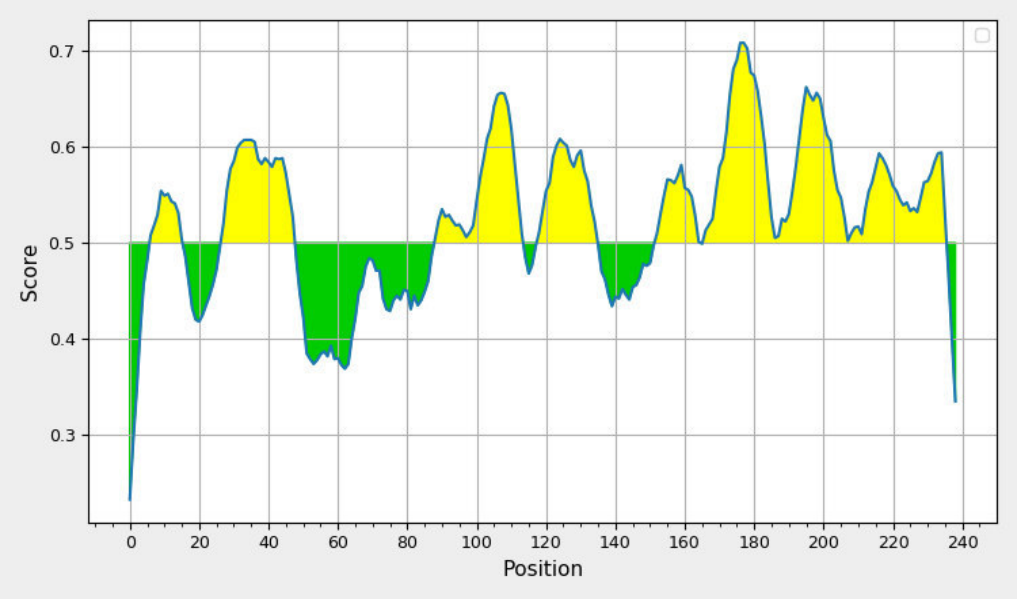


Fig. 4.3 3D model of Protein Variation

A) Epitope prediction by Bepipred Method



B) Epitope prediction by Parker Method

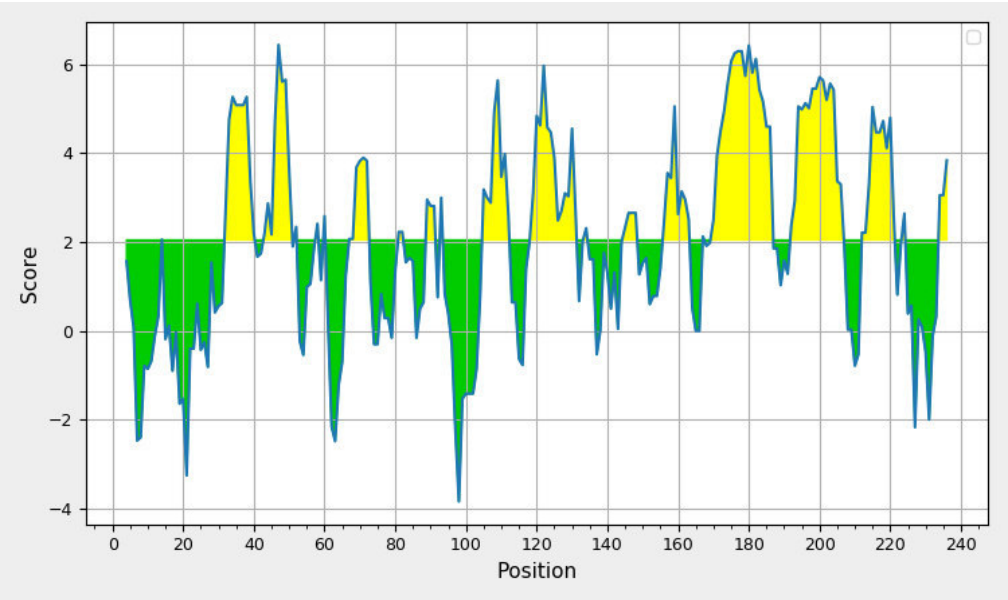


Fig.4.4 Epitope prediction of CPV by Bepipred Method and Parker Method

The nucleotide divergence among the Parbhani Canine parvovirus sequences in this study and other representative isolates of CPV-2a from China, Nigeria and India was studied.

In the table it is evident that the nucleotide divergence within Parbhani CPV-2a isolates was observed to be between 0.007-0.006 per cent (Table 4.4). While the per cent nucleotide homology is between 97 %- 99.2%. From this study isolate no. 1311 has 100 % homology with isolates 1312, 1310. Isolate 1316 has 100% homology with isolates 1314, 1317. The sequence isolate MN810884.1 China Asia showed 100% homology with our isolate 1315 (Table 4.5). Yoldar *et al*., (2019) observed that CPV-2b sequences were having a nucleotide identity of 98.9% with all seven field strains of their study, as well as with other CPV-2b strain. The strains obtained in their study demonstrated 98.7% similarity with CPV/136/ from Italy, obtained in 2000, and 98.9% nucleotide similarity with the rest of the group, consisting of CPV/G133/97/FJ005198 isolated in Germany in 1997, CPV/GR51/ reported from Greece in 2008, CPV/67/ detected in the USA in 2007 and CPV/ Parana/ reported in Brazil in 2009.

4.8 Phylogenetic analysis

A phylogenetic or evolutionary tree is a branching diagram that shows the inferred evolutionary relationships among various biological species or other entities—their phylogeny—based upon similarities and differences in their physical or genetic characteristics.

There are different methods for phylogenetic tree construction but Maximum Likelihood (ML) methods are currently the most commonly used ones in phylogenetic tree construction—as long as sufficient computation power and time are available. For maximum likelihood tree construction, nucleotide/ amino acid substitution model can be tested. This considers different models and selects the one that best fits the input sequence data. Bayesian phylogenetic inference is based on the posterior probability of the tree (the probability of the tree given the data), which is different from the ML method, which searches for the best likelihood of the tree (the probability of the data given the tree). The posterior

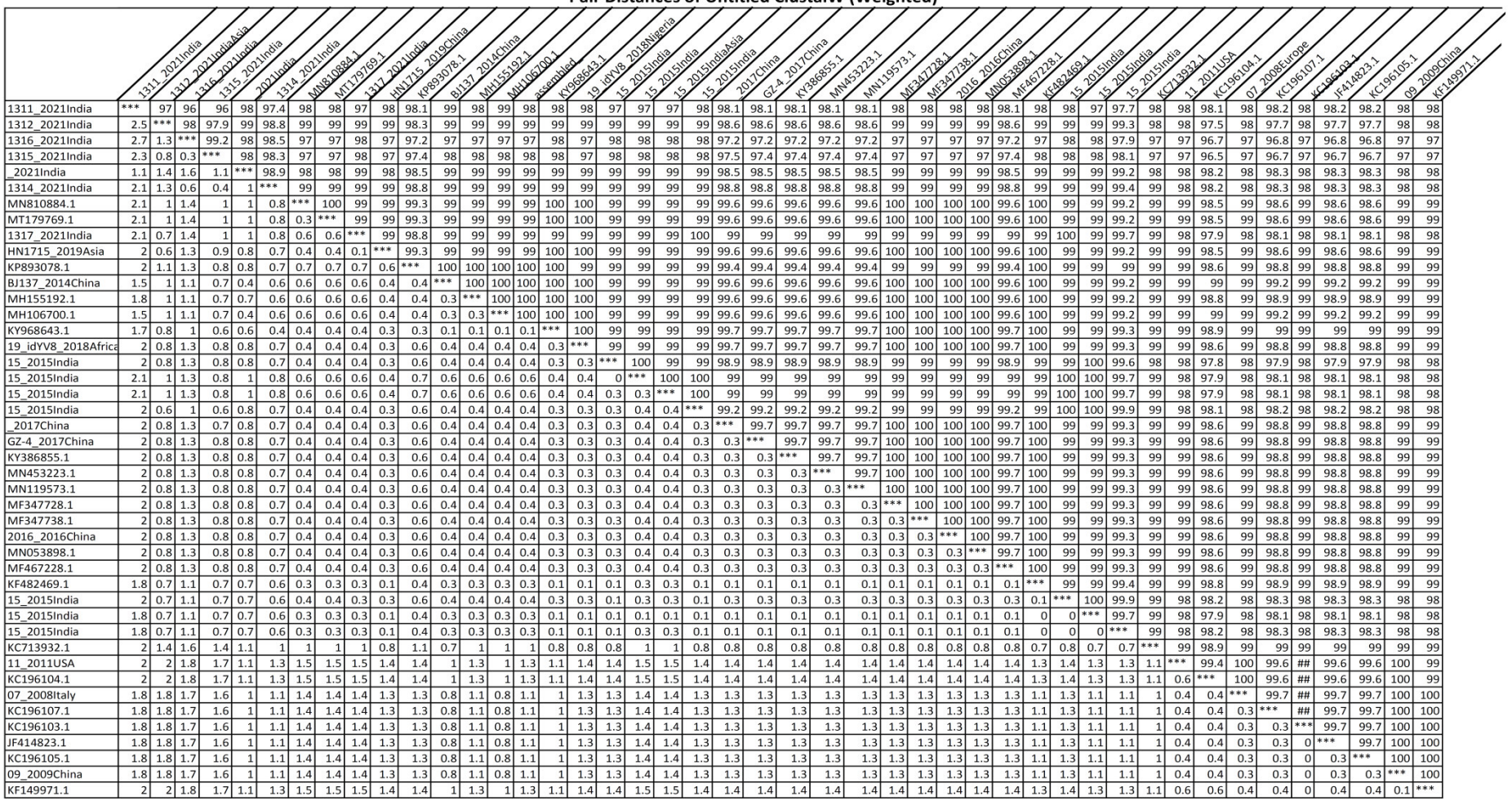
probability is calculated from the prior probability of the phylogeny and the tree likelihood by Bayesian theorem. Bayesian posterior probability usually provides higher support values compared to (non-parametric) bootstrap analysis (Lam *et al.,* 2010). The phylogenetic analysis, we calculated the best nucleotide substitution model for the dataset generated with the sequences of CPV VP2 gene of 2a and 2c strain obtained from GenBank. Phylogenetic analysis was inferred using distance-based (neighbor-joining).

In this study we compared the phylogenetic similarity between CPV strains from clinical cases arrived at TVCC, COVAS, Parbhani. Based on genetic analysis of a fragment of the gene encoding the VP2 protein to perform phylogenetic analysis, the data set of 46 Canine Parvovirus VP2 gene related sequences (07 sequences obtained in the present study and 39 sequences retrieved from Genbank) were aligned using muscle alignment implemented in MEGA7 software.

Total 46 CPV strains were included in a phylogenetic tree. CPV Strains sequenced in this study are highlighted in saffron box (plate 4.7). They are representing 2a strain and are from Indian continent. The reference strains of CPV 2a obtained from NCBI data base were from China, Korea and India all included were of Asian continents, and also from Nigeria of African continents. We represent 2c genotype as a reference stain. All the 2c strains selected were from Asia, USA, America and European continents.

All the 7 (CPV 1310, CPV 1311, CPV 1312, CPV 1314, CPV 1315, CPV 1316, CPV 1317) sequences were closely related to the CPV strains of Indian origin. CPV sequences 1310_2021_India_Asia from this study was closely related to the other Indian CPV isolates are CPV/ PBN /05/15_2015_India@Asia,CN/HN1715_2019@China/Asia and IZSSI_PA1464/19_idYV8_2018@Nigeria#Africa.

Thus study indicates that CPVs of Parbhani, India in our study are phylogenetically closely related to CPV 2a strain of India.



	1311_2021India	1312_2021IndiaAsia	1316_2021India	1315_2021India	_2021India	1314_2021India	MN810884.1	MT179769.1	1317_2021India	HN1715_2019China	KP893078.1	BJ137_2014China	MH155192.1	MH106700.1	assembled	KY968643.1	19_idYV8_2018Nigeria	15_2015India	15_2015India	15_2015IndiaAsia	15_2015India	2017China	GZ-4_2017China	KY386855.1	MN453223.1	MN119573.1	MF347728.1	MF347738.1	2016_2016China	MN053898.1	MF467228.1	KF482469.1	15_2015India	15_2015India	15_2015India	KC713932.1	11_2011USA	KC196104.1	07_2008Europe	KC196107.1	KC196103.1	JF414823.1	KC196105.1	09_2009China	KF149971.1
1311_2021India	***	97	96	96	98	97.4	98	98	97	98	98.1	99	98	99	98	98	98	97	97	97	98	98.1	98.1	98.1	98.1	98	98	98	98	98.1	98	98	97	97.7	98	98	98.1	98	98.2	98	98.2	98	98.2	98	98
1312_2021India	2.5	***	98	97.9	99	98.8	99	99	99	99	98.3	99	99	99	99	99	99	99	99	99	99	98.6	98.6	98.6	98.6	98.6	99	99	99	99	98.6	99	99	99	99.3	98	98	97.5	98	97.7	98	97.7	97.7	98	98
1316_2021India	2.7	1.3	***	99.2	98	98.5	97	97	98	97	97.2	97	97	97	97	98	97	98	98	98	98	97.2	97.2	97.2	97.2	97.2	97	97	97	97	97.2	97	98	98	97.9	97	97	96.7	97	96.8	97	96.8	96.8	97	97
1315_2021India	2.3	0.8	0.3	***	98	98.3	97	97	98	97	97.4	98	98	98	98	98	97	98	98	98	98	97.5	97.4	97.4	97.4	97.4	97	97	97	97	97.4	98	98	98	98.1	97	97	96.5	97	96.7	97	96.7	96.7	97	97
_2021India	1.1	1.4	1.6	1.1	***	98.9	98	98	99	98	98.5	99	99	99	99	99	99	99	99	99	99	98.5	98.5	98.5	98.5	98.5	99	99	99	99	98.5	99	99	99	99.2	98	98	98.2	98	98.3	98	98.3	98.3	98	98
1314_2021India	2.1	1.3	0.6	0.4	1	***	99	99	99	99	98.8	99	99	99	99	99	99	99	99	99	99	98.8	98.8	98.8	98.8	98.8	99	99	99	99	98.8	99	99	99	99.4	99	98	98.2	98	98.3	98	98.3	98.3	98	98
MN810884.1	2.1	1	1.4	1	1	0.8	***	100	99	99	99.3	99	99	99	99	100	100	99	99	99	99	99.6	99.6	99.6	99.6	99.6	100	100	100	100	99.6	100	99	99	99.2	99	99	98.5	99	98.6	99	98.6	98.6	99	99
MT179769.1	2.1	1	1.4	1	1	0.8	0.3	***	99	99	99.3	99	99	99	99	100	100	99	99	99	99	99.6	99.6	99.6	99.6	99.6	100	100	100	100	99.6	100	99	99	99.2	99	99	98.5	99	98.6	99	98.6	98.6	99	99
1317_2021India	2.1	0.7	1.4	1	1	0.8	0.6	0.6	***	99	98.8	99	99	99	99	99	99	99	99	99	100	99	99	99	99	99	99	99	99	99	99	99	100	99	99.7	99	98	97.9	98	98.1	98	98.1	98.1	98	98
HN1715_2019Asia	2	0.6	1.3	0.9	0.8	0.7	0.4	0.4	0.1	***	99.3	99	99	99	99	100	100	99	99	99	99	99.6	99.6	99.6	99.6	99.6	100	100	100	100	99.6	100	99	99	99.2	99	99	98.5	99	98.6	99	98.6	98.6	99	99
KP893078.1	2	1.1	1.3	0.8	0.8	0.7	0.7	0.7	0.7	0.6	***	100	100	100	100	100	99	99	99	99	99	99.4	99.4	99.4	99.4	99.4	99	99	99	99	99.4	100	99	99	99	99	98	98.6	99	98.8	99	98.8	98.8	99	99
BJ137_2014China	1.5	1	1.1	0.7	0.4	0.6	0.6	0.6	0.6	0.4	0.4	***	100	100	100	100	100	99	99	99	99	99.6	99.6	99.6	99.6	99.6	100	100	100	100	99.6	100	99	99	99.2	99	99	99	99	99.2	99	99.2	99.2	99	99
MH155192.1	1.8	1	1.1	0.7	0.7	0.6	0.6	0.6	0.6	0.4	0.4	0.3	***	100	100	100	100	99	99	99	99	99.6	99.6	99.6	99.6	99.6	100	100	100	100	99.6	100	99	99	99.2	99	99	98.8	99	98.9	99	98.9	98.9	99	99
MH106700.1	1.5	1	1.1	0.7	0.4	0.6	0.6	0.6	0.6	0.4	0.4	0.3	0.3	***	100	100	100	99	99	99	99	99.6	99.6	99.6	99.6	99.6	100	100	100	100	99.6	100	99	99	99.2	99	99	99	99	99.2	99	99.2	99.2	99	99
KY968643.1	1.7	0.8	1	0.6	0.6	0.4	0.4	0.4	0.4	0.3	0.3	0.1	0.1	0.1	0.1	***	100	99	99	99	99	99.7	99.7	99.7	99.7	99.7	100	100	100	100	99.7	100	99	99	99.3	99	99	98.9	99	99	99	99	99	99	99
19_idYV8_2018Africa	2	0.8	1.3	0.8	0.8	0.7	0.4	0.4	0.4	0.3	0.6	0.4	0.4	0.4	0.4	0.3	***	99	99	99	99	99.7	99.7	99.7	99.7	99.7	100	100	100	100	99.7	100	99	99	99.3	99	99	98.6	99	98.8	99	98.8	98.8	99	99
15_2015India	2	0.8	1.3	0.8	0.8	0.7	0.4	0.4	0.4	0.3	0.6	0.4	0.4	0.4	0.4	0.3	0.3	***	100	99	99	98.9	98.9	98.9	98.9	98.9	99	99	99	99	98.9	99	99	100	99.6	98	98	97.8	98	97.9	98	97.9	97.9	98	98
15_2015India	2.1	1	1.3	0.8	1	0.8	0.6	0.6	0.6	0.4	0.7	0.6	0.6	0.6	0.6	0.4	0.4	0	***	100	100	99	99	99	99	99	99	99	99	99	99	99	100	100	99.7	99	98	97.9	98	98.1	98	98.1	98.1	98	98
15_2015India	2.1	1	1.3	0.8	1	0.8	0.6	0.6	0.6	0.4	0.7	0.6	0.6	0.6	0.6	0.4	0.4	0.3	0.3	***	100	99	99	99	99	99	99	99	99	99	99	99	100	100	99.7	99	98	97.9	98	98.1	98	98.1	98.1	98	98
15_2015India	2	0.6	1	0.6	0.8	0.7	0.4	0.4	0.4	0.3	0.6	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.4	0.4	***	99.2	99.2	99.2	99.2	99.2	99	99	99	99	99.2	99	100	100	99.9	99	98	98.1	98	98.2	98	98.2	98.2	98	98
_2017China	2	0.8	1.3	0.7	0.8	0.7	0.4	0.4	0.4	0.3	0.6	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.4	0.4	0.3	***	99.7	99.7	99.7	99.7	100	100	100	100	99.7	100	99	99	99.3	99	99	98.6	99	98.8	99	98.8	98.8	99	99
GZ-4_2017China	2	0.8	1.3	0.8	0.8	0.7	0.4	0.4	0.4	0.3	0.6	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.4	0.4	0.3	0.3	***	99.7	99.7	99.7	100	100	100	100	99.7	100	99	99	99.3	99	99	98.6	99	98.8	99	98.8	98.8	99	99
KY386855.1	2	0.8	1.3	0.8	0.8	0.7	0.4	0.4	0.4	0.3	0.6	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.4	0.4	0.3	0.3	0.3	***	99.7	99.7	100	100	100	100	99.7	100	99	99	99.3	99	99	98.6	99	98.8	99	98.8	98.8	99	99
MN453223.1	2	0.8	1.3	0.8	0.8	0.7	0.4	0.4	0.4	0.3	0.6	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.4	0.4	0.3	0.3	0.3	0.3	***	99.7	100	100	100	100	99.7	100	99	99	99.3	99	99	98.6	99	98.8	99	98.8	98.8	99	99
MN119573.1	2	0.8	1.3	0.8	0.8	0.7	0.4	0.4	0.4	0.3	0.6	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.4	0.4	0.3	0.3	0.3	0.3	0.3	***	100	100	100	100	99.7	100	99	99	99.3	99	99	98.6	99	98.8	99	98.8	98.8	99	99
MF347728.1	2	0.8	1.3	0.8	0.8	0.7	0.4	0.4	0.4	0.3	0.6	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.3	***	100	100	100	99.7	100	99	99	99.3	99	99	98.6	99	98.8	99	98.8	98.8	99	99
MF347738.1	2	0.8	1.3	0.8	0.8	0.7	0.4	0.4	0.4	0.3	0.6	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	***	100	100	99.7	100	99	99	99.3	99	99	98.6	99	98.8	99	98.8	98.8	99	99
2016_2016China	2	0.8	1.3	0.8	0.8	0.7	0.4	0.4	0.4	0.3	0.6	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	***	100	99.7	100	99	99	99.3	99	99	98.6	99	98.8	99	98.8	98.8	99	99
MN053898.1	2	0.8	1.3	0.8	0.8	0.7	0.4	0.4	0.4	0.3	0.6	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	***	99.7	100	99	99	99.3	99	99	98.6	99	98.8	99	98.8	98.8	99	99
MF467228.1	2	0.8	1.3	0.8	0.8	0.7	0.4	0.4	0.4	0.3	0.6	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	***	100	99	99	99.3	99	99	98.6	99	98.8	99	98.8	98.8	99	99
KF482469.1	1.8	0.7	1.1	0.7	0.7	0.6	0.3	0.3	0.3	0.1	0.4	0.3	0.3	0.3	0.3	0.1	0.1	0.1	0.3	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	***	99	99	99.4	99	99	98.8	99	98.9	99	98.9	98.9	99	99
15_2015India	2	0.7	1.1	0.7	0.7	0.6	0.4	0.4	0.3	0.3	0.6	0.4	0.4	0.4	0.4	0.3	0.3	0.1	0.3	0.3	0.1	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.1	***	100	99.9	99	98	98.2	98	98.3	98	98.3	98.3	98	98
15_2015India	1.8	0.7	1.1	0.7	0.7	0.6	0.3	0.3	0.3	0.1	0.4	0.3	0.3	0.3	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0	0	***	99.7	99	98	97.9	98	98.1	98	98.1	98.1	98	98
15_2015India	1.8	0.7	1.1	0.7	0.7	0.6	0.3	0.3	0.3	0.1	0.4	0.3	0.3	0.3	0.3	0.1	0.1	0.1	0.3	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0	0	0	***	99	98	98.2	98	98.3	98	98.3	98.3	98	98
KC713932.1	2	1.4	1.6	1.4	1.1	1	1	1	1	0.8	1.1	0.7	1	1	1	0.8	0.8	0.8	1	1	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.7	0.8	0.7	0.7	***	99	98.9	99	99	99	99	99	99	99
11_2011USA	2	2	1.8	1.7	1.1	1.3	1.5	1.5	1.5	1.4	1.4	1	1.3	1	1.3	1.1	1.4	1.4	1.5	1.5	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.3	1.3	1.3	1.1	***	99.4	100	99.6	##	99.6	99.6	100	99
KC196104.1	2	2	1.8	1.7	1.1	1.3	1.5	1.5	1.5	1.4	1.4	1	1.3	1	1.3	1.1	1.4	1.4	1.5	1.5	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.3	1.4	1.3	1.3	1.1	0.6	***	100	99.6	##	99.6	99.6	100	99
07_2008Italy	1.8	1.8	1.7	1.6	1	1.1	1.4	1.4	1.4	1.3	1.3	0.8	1.1	0.8	1.1	1	1.3	1.3	1.4	1.4	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.1	1.3	1.1	1.1	1	0.4	0.4	***	99.7	##	99.7	99.7	100	100
KC196107.1	1.8	1.8	1.7	1.6	1	1.1	1.4	1.4	1.4	1.3	1.3	0.8	1.1	0.8	1.1	1	1.3	1.3	1.4	1.4	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.1	1.3	1.1	1.1	1	0.4	0.4	0.3	***	##	99.7	99.7	100	100
KC196103.1	1.8	1.8	1.7	1.6	1	1.1	1.4	1.4	1.4	1.3	1.3	0.8	1.1	0.8	1.1	1	1.3	1.3	1.4	1.4	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.1	1.3	1.1	1.1	1	0.4	0.4	0.3	0.3	***	99.7	99.7	100	100
JF414823.1	1.8	1.8	1.7	1.6	1	1.1	1.4	1.4	1.4	1.3	1.3	0.8	1.1	0.8	1.1	1	1.3	1.3	1.4	1.4	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.1	1.3	1.1	1.1	1	0.4	0.4	0.3	0.3	0	***	99.7	100	100
KC196105.1	1.8	1.8	1.7	1.6	1	1.1	1.4	1.4	1.4	1.3	1.3	0.8	1.1	0.8	1.1	1	1.3	1.3	1.4	1.4	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.1	1.3	1.1	1.1	1	0.4	0.4	0.3	0.3	0	0.3	***	100	100
09_2009China	1.8	1.8	1.7	1.6	1	1.1	1.4	1.4	1.4	1.3	1.3	0.8	1.1	0.8	1.1	1	1.3	1.3	1.4	1.4	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.1	1.3	1.1	1.1	1	0.4	0.4	0.3	0.3	0	0.3	0.3	***	100
KF149971.1	2	2	1.8	1.7	1.1	1.3	1.5	1.5	1.5	1.4	1.4	1	1.3	1	1.3	1.1	1.4	1.4	1.5	1.5	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.3	1.4	1.3	1.3	1.1	0.6	0.6	0.4	0.4	0	0.4	0.4	0.1	***

Table. 4.4 Phylogenetic analysis maximum-likelihood trees of CPV VP2 amino acid sequences. Trees were constructed using MEGA 7 software with bootstrap support of 1000 replicates. All amino acid sequences were downloaded from the NCBI protein database for analysis, and respective CPV isolates.

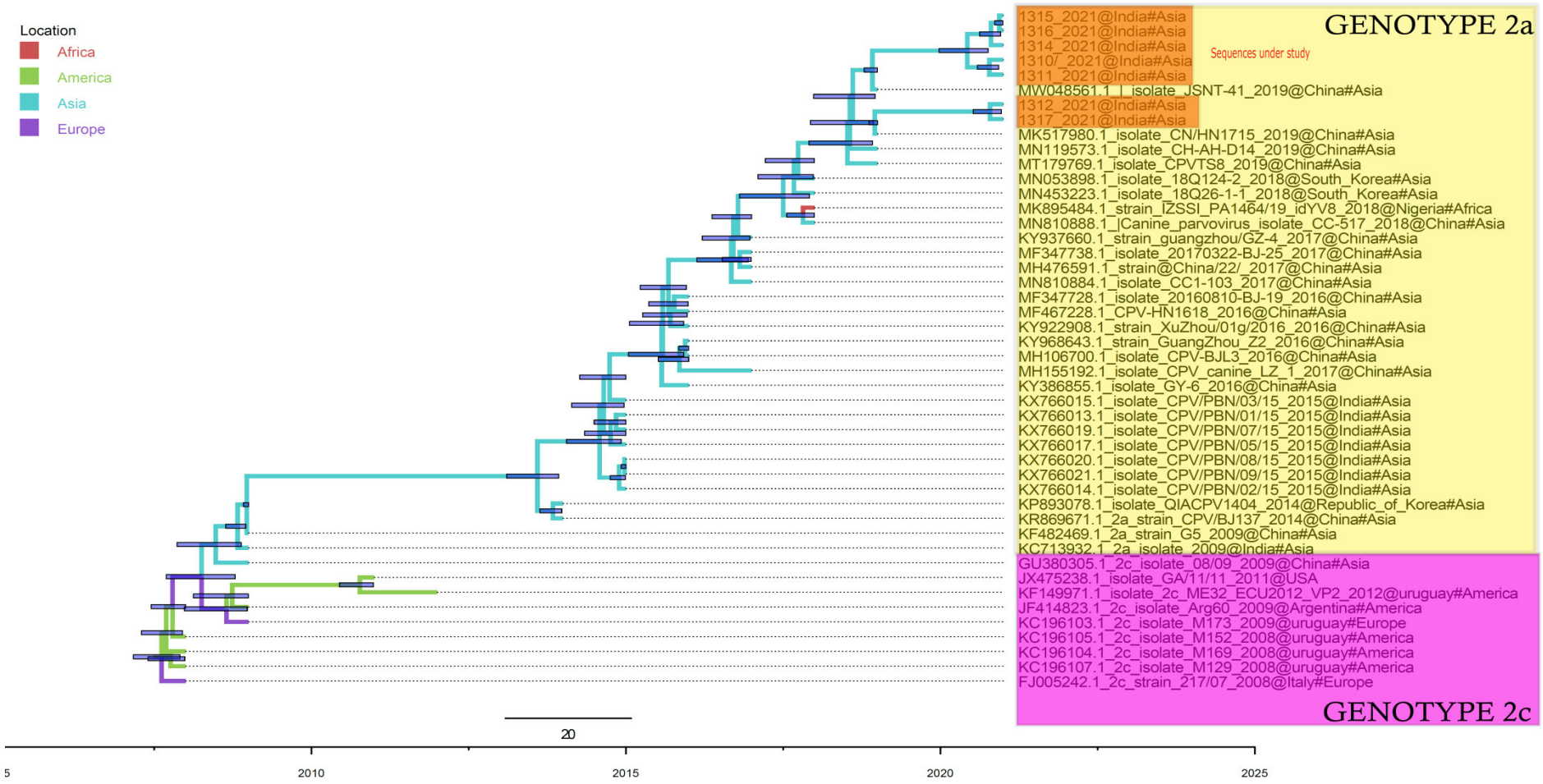


Fig. 1 Location Tree of canine parvo virus.

Strain sequences in the present study are highlighted in Saffron box

Fagbohun *et al*., (2018) also carried out Phylogenetic analysis using maximum likelihood method inferred from partial VP2 sequences showed that the five Nigerian sequences (NGA1, NGA3, NGA4, NGA6 and NGA7) belonged to other CPV-2a from other parts of the world. Zienius *et al*., (2016) carried out a phylogenetic study of CPV and observed that the five Lithuanian CPV VP2 sequences predominantly clustered in a distinct clade and sequences from Lithuania and the sequence from Italy were closely associated with each other.

Table.4.3 Estimation of Evolutionary Divergence between CPV Sequences

Summary and Conclusions

CHAPTER - V

SUMMARY AND CONCLUSION

The Canine parvovirus (CPV-2) infections have emerged as a problem in dogs in recent times around the world. The disease has also been reported in high proportions in dogs in India with casualties even in vaccinated populations. Keeping this in view, present study was undertaken for molecular characterization of Canine parvovirus and its phylogenetic analysis with the following objectives.

1. Molecular detection of Canine Parvo virus from the feces of dogs.
2. Sequencing and Phylogenetic analysis of Canine Parvo virus.

In the present study, fecal samples of dogs suspected of CPV were collected from TVCC Parbhani districts and subjected to PCR for the diagnosis using VP2 and NS1 gene primers of CPV. Out of 25 fecal samples collected from Parbhani district, 23 samples were found positive which revealed a single and uniform band of 747bp size on 1.0 % agarose gel. Primer-dimers were also absent and 5 samples were positive for NS1 gene of CPV, with band size ≈2200 bp.

Total 7 CPV positive PCR products of VP2 gene were subjected to sequence analysis. Based on the sequencing results, the isolates examined in this study were classified as CPV type 2a.

The isolates from Parbhani showed amino acids changes at various positions. The changes were E→Q (glu310gln), E→N (glu426Asn), E→D (glu240asp), D→Y (asp243Tyr), S→T (ser245Thr), H→N(his248Asn), E→V (glu250 Val), R→Q (arg370gln), E→N (glu426Asn), S→Y (ser210Tyr), M→I (met246IIe), Q→P (gln251phe), F→Y(phe267Tyr), E→Q (glu310gln), F→Y(phe267Tyr), Q→P (gln251phe), Q→P (gln251phe), N→D (Asn373asp). Even after the amino acid substitutions changes, all the CPV sequences obtained at Parbhani in the present study were confirmed to be as CPV 2a strain.

The nucleotide divergence of the Canine parvovirus 7 sequences, sequenced in this study and other representative isolates of CPV-2a from China, Nigeria and India, CPV-2c from China, USA, Uruguay, America and Italy

retrieved from geneBank revealed that the nucleotide divergence within Parbhani CPV-2a isolates was observed to be between 0.007-0.006 per cent while the percent nucleotide homology was observed between 97 %- 99.2%. From this study sequences no. 1311 has 100 % homology with sequences 1312 and 1310. Isolate 1316 has 100% homology with isolates 1314, 1317. The sequence isolate MN810884.1 China Asia showed 100% homology with our isolate 1315.

The 3D model of partial VP2 protein indicated protein variation sites in B cell linear epitopes. The two-dimensional protein structures predicted X B cell epitopes in the six and nine epitopes in the sequenced region respectively with antigenicity scores between 0.5 and 0.7 by Bepipred Method and 2 and 6 by Parker Method.

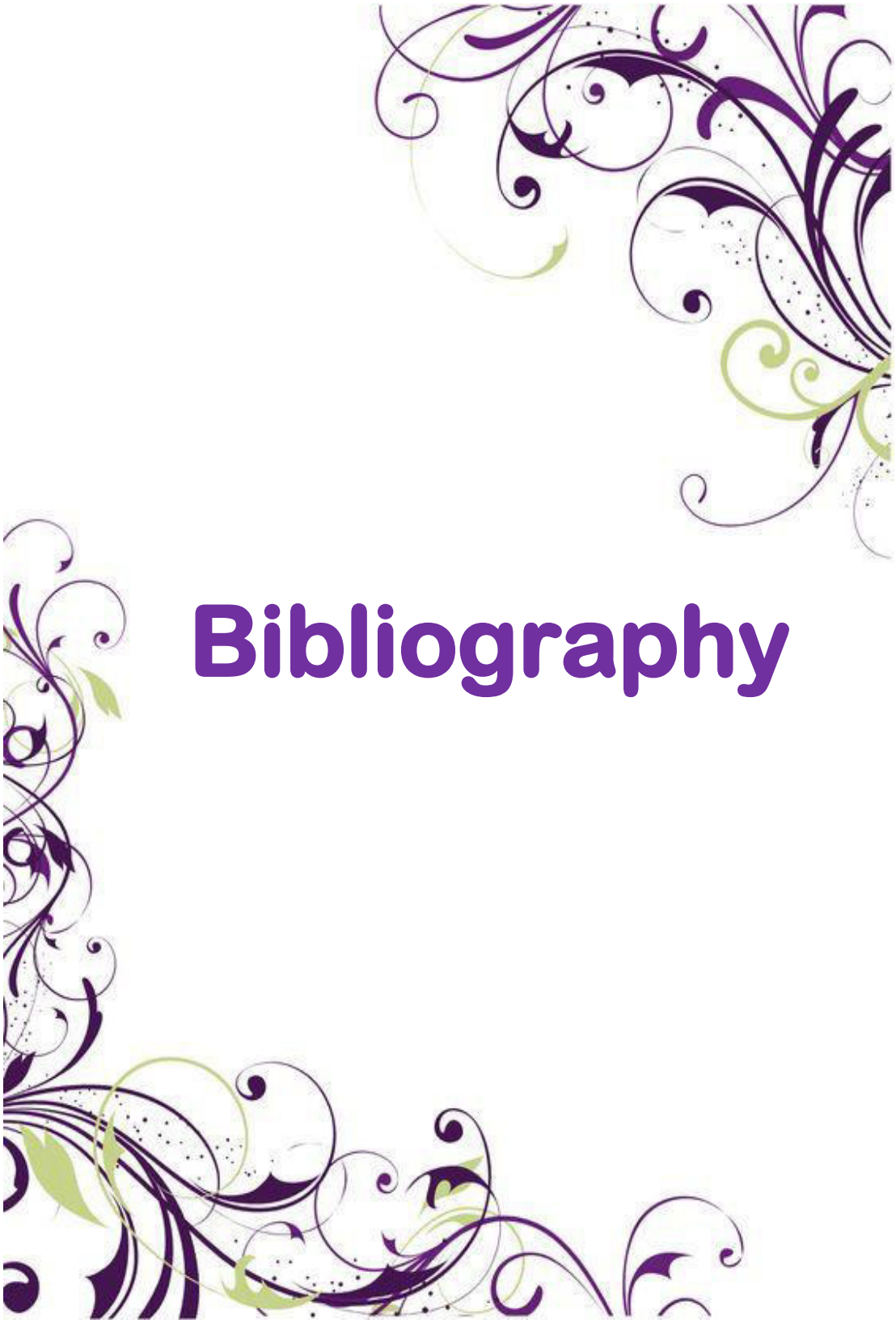
After performing phylogenetic analysis, the data set of 46 Canine Parvovirus VP2 gene related sequences (07 sequences obtained in the present study and 39 sequences retrieved from GenBank) were aligned using muscle alignment implemented in MEGA7 software. All the 7 (CPV 1310, CPV 1311, CPV 1312, CPV 1314, CPV 1315, CPV 1316, CPV 1317) sequences were closely related to the CPV strains of Indian origin. CPV sequences 1310_2021_India_Asia from this study was closely related to the other Indian CPV isolates are CPV/ PBN /05/15_2015_India@Asia, CN/HN1715_2019@China/Asia and IZSSI_PA1464/19_idYV8_2018@Nigeria# Africa. Thus study indicates that CPVs of Parbhani, India in our study are phylogenetically closely related to CPV 2a strain of India.

On the basis of observation of present study investigation following conclusions could be derived.

1. Out of 25 samples processed for CPV, 23 samples were found positive by PCR for VP2 gene primers and 5 samples were positive for NS1 gene primers.
2. Sequence analysis of seven selected samples targeting VP2 gene received that all the Parbhani CPV isolates of this study confirmed as CPV 2a.

3. The maximum nucleotide divergence between Parbhani CPV-2a isolates from this study was between 0.007-0.006 per cent while the percent nucleotide homology was between 97 %- 99.2%.
4. From the Phylogenetic study it was confirmed that nucleotide sequences derived in present study were closely related to the CPV strains of Indian origin. CPV sequences 1310_2021_India_Asia from this study was closely related to the other Indian CPV isolates are CPV/ PBN /05/15_2015_India@Asia,CN/HN1715_2019@China/Asia and IZSSI_PA1464/19_idYV8_2018@Nigeria#Africa.

5. The two-dimensional protein structures predicted X B cell epitopes in the six and nine epitopes in the sequenced region respectively with antigenicity with scores between 0.5 and 0.7 by Bepipred Method and 2 and 6 by Parker Method.



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Vitae

VITAE

The author **Dr. Panchal Mahesh Nagnath** was born on 07th june 1995 at his native place Dhanora (Bk), Tq. Umri, District Nanded of Maharashtra state.

He completed his primary education at Z. P. School, Kundalwadi. He passed Matriculation from Shri Chhatrapati Shivaji High School Sagroli in 2011 and H.S.C. examination in 2013 from Rajashri Shri. Chhatrapati Shahu Sainiki Vidhyalay Sagroli. Thereafter he joined Veterinary profession and completed B.V.Sc. degree from College of Veterinary and Animal Sciences, Parbhani, MAFSU, Nagpur in the year 2019.

Being interested in research and development related to animals he joined postgraduate studies in the discipline of Veterinary Microbiology in the College of Veterinary and Animal Sciences, MAFSU, Parbhani and completed M.V.Sc. degree course in 2021 and submitted his thesis.

He actively participated in Animal Health Camps during his under graduation. During the graduation studies, author represented Maharashtra Animal and Fishery Sciences University, Nagpur in Volleyball at Ashwamedh (Inter University Volleyball Tournaments) held at Vasantrao Naik Marathwada Krishi Vidyapeeth Parbhani in the year. In future he wants to be successful entrepreneur in veterinary profession.

Thesis Abstract

THESIS ABSTRACT

a)	**Title of the thesis (in Capital letters)**	**:**	**MOLECULAR PROFILE OF CANINE PARVO VIRUS**
b)	**Full name of student**	**:**	**PANCHAL MAHESH NAGNATH**
c)	**Name and address of Major Advisor**	**:**	Dr. P. R. Suryawanshi Assistant Professor, Department of Veterinary Microbiology COVAS, Parbhani
d)	**Degree to be awarded**	**:**	M.V. Sc.
e)	**Year of award of degree**	**:**	2021
f)	**Major subject**	**:**	Veterinary Microbiology
g)	**Total number of pages in the thesis**	**:**	61
h)	**Number of words in the abstract**	**:**	212
i)	**Signature of Student**	**:**	
j)	**Signature, Name and address of forwarding authority (HOD/SH).**	**:**	Dr. P.R. Suryawanshi Assistant Professor, Department of Veterinary Microbiology COVAS, Parbhani

ABSTRACT

Canine parvo virus (CPV) infection is a highly infectious viral disease of dogs. Due to increased morbidity and mortality in canines and even in vaccinated dogs the disease has raised the concern. Keeping this in view, present study was carried out for molecular characterisation and phylogenetic analysis of CPV in dogs.

Out of 25 fecal samples that were collected from Parbhani district, 23 samples were found positive which revealed a single and uniform band of 747bp size on 1.0 % agarose gel. Primer-dimers were also absent and 5 samples were positive for NS1 gene of CPV, with band size ≈2200 bp. Seven representative CPV positive PCR products were sequenced. Based on the sequencing results, the isolates examined in this study were classified as CPV type 2a. The nucleotide divergence of the Canine parvovirus 7 sequences, and nucleotide divergence within Parbhani CPV-2a isolates was observed to be between 0.007-0.006 per cent while the percent nucleotide homology was observed between 97 %- 99.2%.

Performing B cell linear epitope predictions antigenicity with scores between 0.5 and 0.7 was observed by Bepipred Method and 2 and 6 by Parker Method.

On the basis of Phylogenetic analysis study indicates that CPVs of Parbhani, India in our study were phylogenetically closely related to CPV 2a strain of India.

प्रबंध सारांश

a)	**प्रबंधाचे शीर्षक**	:	कॅनाइन पारवो व्हायरसचे आण्विक प्रोफाइल
b)	**विद्यार्थ्यांचे पूर्ण नाव**	:	पांचाळ महेश नागनाथ
c)	**प्रमुख मार्गदर्शकाचे नाव व पत्ता**	:	डॉ. प्रशांत रा. सुर्यवंशी सहाय्यक प्राध्यापक, सूक्ष्मजीवशाश्त्र विभाग पशुवैद्दकिय व पशुविज्ञान महाविद्यालय परभणी
d)	**प्रदान करण्यात येणारी पदवी**	:	एम. व्ही. एस सी
e)	**पदवी प्रदान करण्याचे वर्ष**	:	२०२१
f)	**मुख्य विषय**	:	पशुवैद्यकीय सूक्ष्मजीवशाश्त्र
g)	**प्रबंधातील पानांची एकूण संख्या**	:	61
h)	**सारांशामधील शब्दांची संख्या**	:	212
i)	**विद्यार्थ्यांची स्वाक्षरी**	:	
j)	**पाठविणाऱ्या अधिकाऱ्याची स्वाक्षरी, नाव व पत्ता (विभागप्रमुख)**	:	डॉ. प्रशांत रा. सुर्यवंशी सहाय्यक प्राध्यापक, सूक्ष्मजीवशाश्त्र विभाग पशुवैद्दकिय व पशुविज्ञान महाविद्यालय परभणी

सारांश

कॅनाइन पारवो विषाणू (CPV) संसर्ग हा कुत्र्यांचा एक अत्यंत संसर्गजन्य विषाणूजन्य रोग आहे, ज्यामुळे लसीकरण केलेल्या कुत्र्यांमध्येही कुत्र्यांमधील विकृती आणि मृत्यूचे प्रमाण वाढले आहे. हे लक्षात घेऊन, कुत्र्यांमधील CPV चे आण्विक वैशिष्ट्य आणि फायलोजेनेटिक विश्लेषणासाठी सध्याचा अभ्यास केला गेला.

परभणी जिल्ह्यातून गोळा केलेल्या 25 विष्ठा नमुन्यांपैकी 23 नमुने पॉझिटिव्ह आढळून आले ज्यामध्ये 1.0% ॲग्रोज जेलवर 747bp आकाराचा सिंगल आणि एकसमान बँड दिसून आला. प्राइमर-डायमर देखील अनुपस्थित होते आणि 5 नमुने CPV च्या NS1 जनुकासाठी सकारात्मक होते, बँड आकार ≈2200 bp सह. सात प्रतिनिधी सीपीव्ही पॉझिटिव्ह पीसीआर उत्पादने अनुक्रमित केली गेली. अनुक्रम परिणामांवर आधारित, या अभ्यासात तपासलेल्या आयसोलॅट्सचे CPV प्रकार 2a म्हणून वर्गीकरण करण्यात आले. परभणी CPV-2a आयसोलेट्समधील कॅनाइन पारवो व्हायरस 7 अनुक्रमांचे न्यूक्लियोटाइड विचलन आणि न्यूक्लियोटाइड विचलन 0.007-0.006 टक्के दरम्यान आढळून आले, तर न्यूक्लियोटाइड टक्के समरूपता 97% - 99.2% दरम्यान आढळून आली.

0.5 आणि 0.7 दरम्यानच्या स्कोअरसह बी सेल रेखीय एपिटोप अंदाज प्रतिजैविकता पार पाडणे बेपिप्रेड पद्धतीद्वारे आणि 2 आणि 6 पार्कर पद्धतीद्वारे पाहिले गेले.

फायलोजेनेटिक विश्लेषणाच्या आधारे अभ्यास असे सूचित करतो की आमच्या अभ्यासात परभणी, भारतातील CPVs हे भारताच्या CPV 2a स्ट्रेनशी फायलोजेनेटिकदृष्ट्या जवळचे संबंधित होते.