A STUDY ON *IN-VITRO* CROSS-NEUTRALISATION ASSAY FOR IDENTIFYING ANTIGENIC RELATIONSHIP OF CANINE PARVOVIRUS TYPES

Thesis

Submitted to Guru Angad Dev Veterinary and Animal Sciences University in partial fulfilment of the requirements for the degree of

MASTER OF VETERINARY SCIENCE
in
VETERINARY MICROBIOLOGY
(Minor Subject: Animal Biotechnology)

By

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2020

CERTIFICATE I

This is to certify that the thesis entitled, **"A study on *in-vitro* cross-neutralisation assay for identifying antigenic relationship of canine parvovirus types"** submitted for the degree of **M.V.Sc.,** in the subject of **Veterinary Microbiology** (Minor subject: **Animal Biotechnology**) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Karman Kour (L-2018-V-64-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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ACKNOWLEDGEMENT

This thesis has become possible with the kind support and help of many individuals to whom I would like to express my sincere gratitude.

Foremost, I would want to offer this endeavour to my major advisor, **Dr, Gurpreet Kaur,** Assistant Professor, Department of Veterinary Microbiology. I feel lucky and blessed to have been chosen as her student. I am indebted to her for her guidance and constant supervision in each and every step of my research work and my thesis completion. Her sincerity towards her work and time management has always motivated me and helped me evolve as a person and as a researcher.

To the worthy members of my advisory committee namely **Dr P.N. Dwivedi**, Professor, Veterinary Microbiology, **Dr. Ramneek,** Professor cum Director, College of Biotechnology and **Dr. T.S. Rai**, Professor-cum-Head (Dean PG's Nominee), Department of Veterinary Microbiology who manifested their distinguished skills and talents in their fields as seen in their ways of correction and ideas shared.

It would not have been possible for me to work efficiently if I would not have been blessed with the most amazing and supportive team of teachers. I express my heartfelt gratitude to the teaching staff of Department of Veterinary Microbiology **Dr. A.K, Arora**, Professor, Department of Veterinary Microbiology, **Dr. Deepti Narang**, Professor, Department of Veterinary Microbiology, **Dr. Paviter Kaur**, Assistant Professor, Department of Veterinary Microbiology and I would like to place on record my deepest sense of gratitude to **Dr. Mudit Chandra**, Assistant Scientist, Department of Veterinary Microbiology for his timely advices, constant guidance, valuable comments and provisions that benefitted me much in the completion of this study.

My sincere thanks to our respected Dean PGS **Dr. Sanjeev Kumar Uppal**, Professor, Department of Veterinary Medicine for his worthy support and cooperation throughout our degree years.

Immeasurable appreciation and deepest gratitude for the help and support are extended to **Dr. Sreekala S Mohandas**, Ph.D. scholar, Veterinary Microbiology, my worthy seniors, **Dr. Sailakshmikanth Katrapati**, Junior Research Fellowship, Veterinary Microbiology, **Navjot Kaur**, Junior Research Fellowship, Veterinary Microbiology for always guiding me and being there for me throughout my time of study here.

I feel obliged in taking the opportunity to sincerely thank **Dr. Amritpal Sethi**, Professor-cum-Head, Department of Veterinary Nutrition and **Dr. Prahlad Singh**, Professor cum Head, Department of Veterinary Gynaecology for bestowing their expertise and guidance and for their constant support and help in all circumstances.

Most importantly my heart is filled with love and respect for my family who have always believed in me and aspire me to give my best come what may. My father ***Dr. Bikram Singh*** *for being a moral support, a constant guide and a teacher who held my hand in every difficulty I faced. My mother* ***Mrs. Ranbir Kour*** *who has been the pillar of my strength throughout my life. My siblings* ***Chanandeep Kour*** *and* ***Abheet Singh*** *without whose genuine criticism I couldn't ever strive hard and achieve my goals.*

Apart from my teachers and my family, there are some special names without whom this would not have been possible. My seniors ***Dr. Himasri Das, Dr. Jasleen Kaur, Dr. Asiya Mushtaq, Dr. Sundus Gazal, Dr. Isha Sekhri. My batchmates Harnoor Kaur, Parikshit Singh, Jasleen Kour, Arpit Sachdeva, Surbhi Rometra, Savleen Kour*** *and* ***Harshveer Singh Grewal****. Also, my juniors* ***Ayushi Langeh, Ravneet Kaur, Roop Kiran Malhi, Arzoo*** *and* ***Harjot Chopra****. All of you have made my two year M.V.Sc. degree most cherishable and memorable.*

The help rendered by the office staff ***Ms. Ravneet, Mr Uday, Mr Harjeet, Mr Charanjeet*** *and* ***Mr. Amit*** *is utterly acknowledged. Lastly and importantly, my sincere thanks to* ***Mr. Gurdeep Singh (ALPS)*** *for his kind help for helping me finalise the manuscript. I am also thankful to DBT for funding my project BT/ADV/Canine Health/TANUVAS/2017-18 and make this project a success.*

I acknowledge each one of you with all my heart.

Place: Ludhiana

Date: 20.08.2020 ***(Karman Kour)***

Title of the Thesis	:	**A study on *in-vitro* cross-neutralisation assay for identifying antigenic relationship of canine parvovirus types**
Name of the Student	:	Karman Kour
Admission No.		L-2018-V-64-M
Major Subject	:	Veterinary Microbiology
Minor Subject	:	Animal Biotechnology
Name and Designation of Major Advisor	:	Dr. Gurpreet Kaur Assistant Professor
Degree to be awarded	:	M.V.Sc.
Year of award the Degree	:	2020
Total pages of Thesis	:	85 + VITA
Name of University	:	Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana – 141 004 (Punjab), India

ABSTRACT

Canine Parvovirus (CPV) causing hemorrhagic gastroenteritis in dogs is much prevalent throughout India and worldwide. The virus is non-enveloped linear single stranded DNA virus, having approximately about 5,323 bases. The virus comprises of structural proteins (VP-1, VP-2 and VP-3) forming the capsid and two non-structural proteins (NS-1 and NS-2). VP2 is the immunodominant protein of the virus and major capsid protein responsible for various antigenic types of CPV. The antigenic types of CPV prevalent worldwide are CPV 2, CPV 2a, CPV 2b and CPV 2c. Commercial vaccines for CPV are available but despite vaccination dogs are suffering from the disease. One reason for this is attributed to the difference in the antigenic types in vaccines and field strains. Therefore, it is utmost important to identify and understand the prevailing antigenic type/s of CPV in various geographical regions of India and to study the cross protectivity of the various antigenic types prevailing using cross-neutralisation assay. Hence the first objective was aimed at identifying the prevailing antigenic type/s of CPV in northern region of India using real-time PCR (RT PCR). For this the rectal swabs were collected from dogs suspected of having the disease from various regions of northern India viz. Punjab (n=119), Assam (n=36), Delhi (n=11), Jammu (n=6) and Chandigarh (n=6). The virus was detected using RT PCR wherein the probes specific for the antigenic type provided a clear picture of prevailing CPV type/s of the virus in different geographical regions. Out of the total 178 samples, 27 were positive for CPV 2, 86 were positive for CPV 2a and 20 were positive for CPV 2b. Moreover it was found that some of the samples from dogs were positive for more than one antigenic type of the virus. For the second objective of cross-neutralisation assay, random isolate of CPV 2a and CPV 2b were grown in bulk in MDCK cell line. The virus was then purified by ultracentrifugation and hyperimmune sera for both CPV 2a and CPV 2b was raised in Rabbits. After confirming the hyperimmune sera by indirect ELISA, 100TCID$_{50}$ was calculated for both CPV 2a and CPV 2b to be used in cross-neutralisation assay. It was concluded that the titre of serum 2a at which it can neutralise CPV 2b was 4096 and the titre of serum 2b at which it could neutralise CPV 2a was 2048. The titre of both the serum for the homologous virus type was found to be higher i.e. 8192.

Keywords: Canine parvovirus, real-time PCR, cell culture, antigenic typing, hyperimmune serum, indirect ELISA, TCID$_{50}$, cross-neutralisation assay

Signature of Major Advisor **Signature of the Student**

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LIST OF ABBREVIATIONS

%	:	Percent
°C	:	Degree Celsius
µg	:	Microgram
µl	:	Microliter
AAV	:	adeno-associated viruses
AC	:	Antigen Capture
Ala	:	Alanine
Asn	:	Asparagine
Asp	:	Aspartic acid
BFPV	:	blue fox parvovirus
bp	:	Base pair
CDV	:	Canine Distemper Virus
CIE	:	Counter Immuno electrophoresis
COVS	:	College of Veterinary Sciences
CPE	:	Cytopathic Effect
CPT	:	Cone Penetration testing
CPV	:	Canine parvovirus type 2
CRFK	:	Crandell-Rees Feline kidney
Ct	:	Cycle threshold
DAS	:	Double Antibody sandwich
DHPPi	:	Canine distemper virus, canine adnovirus, canine parvovirus, canine parainfluenza virus
DK	:	Dog kidney
DMEM	:	Dulbecco's Minimum Essential Media
DNA	:	Deoxyribonucleic acid
dNTP	:	Dinucleoside triphosphate
EDTA	:	Ethylinediaminetetraacetic acid
ELISA	:	Enzyme-linked immunosorbent assay
EM	:	Electron Microscopy
F	:	Female
FAT	:	Fluorescent antibody test
FBS	:	Fetal Bovine Serum
FCS	:	Fetal Calf serum
FPV	:	Feline panleukopenia virus
FRET	:	fluorescence resonance energy transfer
g	:	gram
glu	:	Glutamic acid
Gly	:	Glycine

GM	:	Growth medium
GSD	:	German Shephard Dog
HA	:	Haemagglutination
HI	:	haemagglutination inhibition
HRPO	:	Horseradish peroxidase
hrs	:	Hours
IC	;	Immunochromatography
IFA	:	Immunofluorescence assay
Ig	:	Immunoglobulin
ile	:	Isoleucine
kb	:	Kilo bases
kDa	:	Kilo dalton
KLH	:	Keyhole limpet hemocyanin
LAMP	:	loop-mediated isothermal amplification
LAT	:	Latex Agglutination Test
Leu	:	Leucine
LNA	:	Locked Nucleic Acid
M	:	Male
m.u.	:	map units
MABs	:	monoclonal antibodies
MCV	:	minute virus of canine
MDA	:	Maternally derived antibodies
MDCK	:	Madin Darby Canine Kidney
MEM	:	Minimum Essential Media
Met	:	Methionine
MEV	:	Mink enteritis virus
mg	:	Milligram
MGB	:	Minor Groove Binder
min	:	Minutes
ml	:	Mililitre
MLV	:	Modified Live virus
MM	:	Master Mix
MM	:	Maintenance media
mRNA	:	Messenger Ribonucleic acid
NFW	:	Nuclease Free Water
nm	:	Nanometer
nmol	:	Nanomole
NRCE	:	National Research Centre on Equines
NS	:	Non-structural

nt	:	Nucleotides
Nt	:	Neutralizing
OD	:	Optical density
p.i.	:	Post infection
PBS	:	Phosphate Bufered saline
PCI	:	Phenol Chloroform isoamyl alcohol
PCR	:	Polymerase chain reaction
PNA	:	Peptide Nucleic Acid
PVNR TVU	:	P.V. Narsimha Rao, Telangana Veterinary University
qPCR	:	Quantitative Polymerase Chain Reaction
RDVP	:	Racoon Dog parvovirus
RE	:	Restriction Enzyme
RFU	:	Relative Fluorescence Unit
RNA	:	Ribonucleic acid
Rpm	:	Revolutions per minute
RPMI	:	Rosewell Park Memorial Institute
RPV	:	Raccoon parvovirus
RT-PCR	:	Reverse-Transcriptase Polymerase chain reaction
SDS	:	Sodium Dodecyl sulphate
sec	:	Seconds
SN	:	serum neutralization
SNP	:	Single nucleotide polymorphisms
SVN	:	Serum Virus Neutralisation
TBE	:	Tris-borate-EDTA
TCID	:	Tissue Culture Infective Dose
TD	:	Touchdown
thr	:	Threonine
trp	:	Tryptophan
Tyr	:	Tyrosine
U.K.	:	United Kingdom
UNG	:	Uracil-N-Glycolase
Val	:	Valine
VI	:	Virus Isolation
VNT	:	Virus Neutralization Test
VP	:	viral protein

CHAPTER I

INTRODUCTION

"The best therapist has fur and four legs".

Puppies are the epitome of youth, full of vim and vigor, they exude joy and energy in everything they do. Unfortunately, it's their very youth that makes puppies susceptible to certain conditions that may be life-threatening. An immature immune system and bad genetics can all add up to puppy diseases and conditions. There are many diseases that come in conflict with the average dog today. If no precautions are taken to prevent these diseases, chances are the dog is more than likely to contract one. One of the most common among them is called Canine Parvovirus found in puppies which are 6 to 20 weeks old. (Miranda *et al* 2015)

Parvoviruses are nonenveloped, single-stranded DNA viruses belonging to the genus Protoparvovirus, member of the Parvoviridae family, that has been included within the species Carnivore protoparvovirus 1, together with Feline panleukopenia virus (FPV), Mink enteritis virus (MEV) and Raccoon parvovirus (RPV), according to the International Committee on Taxonomy of Viruses (Tijssen *et al* 2011). It is the most important enteric virus infecting canids and is designated as Canine parvovirus type 2 (CPV-2). Two distinct parvoviruses are known to infect dogs—the pathogenic CPV-2 and CPV-1 or the minute virus of canine (MVC). CPV-2, the causative agent of acute hemorrhagic enteritis and myocarditis in dogs, is one of the most important pathogenic viruses with high morbidity (100%) and frequent mortality up to 10% in adult dogs and 91% in pups. CPV-2 is the etiologic agent of a contagious disease, mainly characterized by clinical gastroenteritis signs in younger dogs. This virus that causes the disease "parvo", first emerged among dogs in Europe around 1976. By 1978 the virus had spread unchecked, causing a worldwide epidemic of myocarditis and inflammation in the intestines (gastroenteritis) and could infect domestic dogs, spreading in the global dog population within 2 years. This original type, was rapidly replaced by two antigenic variants, types 2a and 2b (Parrish *et al* 1985, 1991), and in 2000 a third type CPV-2c was detected (Buonavoglia *et al* 2001). Canine parvovirus (CPV) is responsible for acute gastroenteritis in pups, with a high rate of mortality (Carmichael and Binn, 1981). We now know the virus is not limited to dogs, but is capable of causing infections in wild canines such as coyotes and wolves, and other

wild animals, including foxes, raccoons and skunks. Since its emergence in 1978, it was and remains a common and important cause of morbidity and mortality in young dogs.

Parvovirus infection of dogs results in systemic infection following oropharyngeal entry of the virus. Intestinal lesions in affected dogs result from infection and destruction of enterocytes populating the intestinal crypts, with subsequent mucosal collapse, maldigestion and malabsorption diarrhea. This virus has an affinity for rapidly dividing cells of the intestine and causes acute enteritis with intestinal crypt necrosis and villus atrophy. Dogs and cats encounter parvoviruses in fomites from body fluids contaminated with fecal matter through direct contact with infected animal hence rendering fecal sample as the best sample to study parvovirus.

By sequence analysis CPV-2 appeared to be closely related to feline parvovirus (FPV) and also to parvoviruses from raccoons, minks, and arctic foxes (Parrish, 1999; Truyen, 2006), with the nucleotide variation from FPV being lower than 0.5%. In the 1980s the original CPV-2 was completely replaced by new antigenic variants designated CPV-2a and CPV-2b, and the original virus was no longer found to be present in the canine population and existed only in the vaccine formulations. The CPV capsid consists of 60 protein subunits that assemble to form a 26 nm icosahedron. The viral capsid is formed by structural proteins VP1, VP2 and VP3, with VP2 being predominant. Approximately 90% of the capsid is made up of a 67 kDa protein known as viral protein 2 (VP2) with the remaining 10% being composed of the 83 kDa viral protein 1 (VP1). The amino acid sequence of VP1 is almost identical to that of VP2 with the exception of a 143 amino acid sequence at the amino-terminal domain. Protein folding of VP1 sequesters the amino-terminal domain; however, when exposed to high temperatures or acidic pH, VP1 changes its conformation, exposing the amino-terminus. VP3 is a proteolytic product formed by removal of part of the amino terminus of VP2. The N-terminus contains a neutralization site conserved in different viral isolates which in the form of synthetic peptides is able to induce neutralizing antibodies and protection in the host (Langeveld *et al* 1994a; Langeveld *et al* 1994b). There are at least five or six amino acid changes between the variants CPV-2a/b and the original CPV-2 in the VP2 capsid protein (Parrish *et al* 1991), while the variant CPV-2a differs from the variant

CPV-2b only in the change 426-Asn→Asp within the major antigenic site of the capsid (Parrish *et al* 1991; Parrish *et al* 1998). Soon after the appearance of the CPV-2a/b variants, a number of additional, unusual mutations affecting important residues of the capsid protein VP2 of CPV were recognized, suggesting that CPV is still evolving (Buonavoglia *et al* 2001; Ikeda *et al* 2000; Truyen *et al* 2000).

Parvovirus can be detected with a commercially available fecal enzyme-linked immunosorbent assay (ELISA) but due to intermittent and brief shedding of the virus, fecal ELISAs can have false-negative results. Therefore, other laboratory diagnosis of canine parvovirus infection include hemagglutination of pig, cat, or rhesus monkey red blood cells (pH 6.5, 4°C) by virus present in fecal extracts, and the specificity of this hemagglutination is determined by titrating the sample in parallel in the presence of normal and immune dog serum. Fecal samples from dogs with acute enteritis may contain thousands of hemagglutinating units of virus, reflecting very high titers of virus. Other diagnostic techniques like Electron microscopy, virus isolation, and amplification of viral DNA using PCR assay on fecal samples are also used for laboratory confirmation of clinical diagnosis. Retrospective diagnosis can be done with serology, typically using the immunoglobulin IgM and/or IgG-capture enzyme-linked immunosorbent assay on paired sera. PCR can be used to confirm an ELISA result and to differentiate the viral strain.

To arrive to the best diagnostic technique, scientists have compared several techniques overtime. Immunochromatography (IC), haemagglutination (HA), virus isolation (VI), conventional and real-time PCR are few of them. Comparison between these different techniques revealed that real-time PCR is more sensitive than HA and conventional gel-based PCR, allowing to detect low viral titers of CPV-2 in infected dogs (Decaro *et al* 2005).

Real-time PCR has engendered wider acceptance of the PCR due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination. There are currently five main chemistries used for the detection of PCR product during real-time PCR (Mackay *et al* 2002). These are the sequence-unspecific DNA labeling dyes (SYBR green), primer-based technologies (AmpliFluor, Plexor, Lux primers), and techniques involving double-labeled probes, comprising hybridization (molecular beacon) and hydrolysis (TaqMan, CPT, LNA,

and MGB) probes. Among the most commonly used real-time PCR chemistries are TaqMan probes and SYBR green (Gasparic *et al* 2010).

Canine parvovirus (CPV) is an important pathogen in domestic dogs, and the original antigenic types CPV-2 and its variants, CPV-2a, 2b and 2c, are prevalent worldwide. A multiplex TaqMan real-time PCR method aids in the detection and differentiation of four antigenic types of CPV. A set of primers and probes, CPV-305F/CPV-305R and CPV-2-305P (for CPV-2)/CPV-2a-305P (for CPV-2a, 2b and 2c), was able to differentiate CPV-2 and its variants (CPV-2a, 2b and 2c). Another set of primers and probes, CPV-426F/CPV-426R and CPV-2-426P (for CPV-2 and 2a)/CPV-2b-426P (for CPV-2b)/CPV-2c-426P (for CPV-2c), was able to differentiate CPV-2a (Pollock and Parrish, 1985), CPV-2b, and CPV-2c. With these primers and probes, the multiplex TaqMan real-time PCR assay detects effectively and differentiates CPV-2, 2a, 2b and 2c by two separate real-time PCRs. No cross reactivity with canine distemper virus, canine adenovirus, and canine coronavirus was observed. The detection limit of the assay was found to be 10^1genome copies/μl for CPV-2, CPV-2a, CPV-2b, and 10^2copies/μl for CPV-2c. Also, the multiplex real-time PCR has 100% agreement with DNA sequencing. Therefore, it provides a sensitive assay that simultaneously detects and differentiates four antigenic types of CPV and this method can also be used for quantification of CPVs viral genome (Sun *et al* 2018).

Vaccination is the most cost effective and ideal method to control the canine parvovirus infections in canines. Both live attenuated and inactivated vaccines are available to control the disease in animals. In spite of large scale vaccination to control the disease in dogs, the disease has been reported both in vaccinated and the unvaccinated dogs (Sukdeb Nandi, Manoj Kumar, Tapas Kumar Mohapatra and Chintu Ravishankar). Also, there is continued incidence of parvoviral enteritis which is believed to be partly due to the virus's capability to "reinvent" itself and evolve into new more virulent and resistant subspecies. This evolution of the virus raises questions about the efficacy of some vaccines, as there are concerns that the vaccines used currently to prevent CPV infection in dogs may fail to effectively protect pups against the new CPV antigenic variants (Truyen, 2006). We know that since the identification of canine parvovirus type 2, three variants have subsequently been

observed differing from the historical CPV-2 and each other by 1–2 amino acids only. As a result there has been considerable research into differential diagnostics, with some researchers indicating there is a need for new vaccines containing different strains of CPV-2 as some studies also indicated that the animals vaccinated for CPV were also found positive for the disease (Kushwaha, 2018; Wilson *et al* 2014).

Analysis of canine parvovirus (CPV) isolates with a panel of monoclonal antibodies showed that after 1986, most viruses isolated from dogs in many parts of the world differed antigenically from the viruses isolated prior to that date. Phylogenetic analysis showed a progressive evolution away from the original CPV type (Parrish *et al* 1991).

In spite of latest development in the field of virology, immunology, biotechnology, genetics, genomics, proteomics etc. many aspects about CPV-2 infections that may help in both preventing vaccination failure and in controlling the disease are still unexplored. There are differences in opinion about the efficacy of existing CPV vaccine in controlling the new variants of CPV and a variety of vaccination regimes are adopted by the veterinarians against the CPV infections. Some are of the opinion that the current vaccine based on CPV-2 is still effective against all the CPV variants [Spibey 2008 and Larson 2008]. Others opine that as there is no incidence of CPV-2 outbreaks now-a-days, the vaccine strain (CPV-2) must be replaced by new variants of CPV-2a/2b/2c based on the prevalence disease in a particular region [Decaro,2007; Decario and Desario 2008]. With a better understanding of the disease, cross protective activity of the different mutants and various causes of vaccine failure, it would be possible for the veterinary practitioners to discharge the best possible management and the immunoprophylactic measures which in turn help in the prevention and the control of the CPV infections in dogs.

The neutralization test is the "gold standard" for detection of antibodies against a few viruses. Though, ELISAs are being used more, neutralization tests with a prolonged (up to 24 hours) virus serum incubation period are generally more sensitive. The serum virus neutralization (SVN) assay is a serological test to detect the presence and magnitude of functional systemic antibodies that prevent infectivity of a virus. It is a highly sensitive and specific test that may be applied to viruses to measure the titer of neutralizing antibodies post-infection or after vaccination.

Conventional SVN methods performed in vitro are based on inhibition of virus infectivity in cell culture in the presence of neutralizing antibodies. Titer determination is done and is based on the presence or the absence of cytopathic effect or the evidence of viral infection using an immunoreactive technique. The SVN assay is relatively inexpensive using standard laboratory equipment although it requires cell culture, more time and labor, and technical skill to conduct the assay compared to other serological methods. This test is useful to evaluate the level of serological cross-reactivity between vaccine antisera and the virus subtypes that may correlate with cross-protection in the host.

Antigenic relationships among the original CPV-2 and the variants CPV-2a, CPV-2b and CPV-2c when evaluated by haemagglutination inhibition (HI) and serum neutralization (SN) test, it is observed that former test is not adequate to evaluate the real protective immunity of dogs in particular against the antigenic variants (Cavalli *et al* 2008).

Hence, keeping in view the above issues, the objectives of this study was planned as follows:

1. To identify antigenic types of CPV using real-time PCR.
2. To identify antigenic relationship of canine parvovirus types using *in-vitro* cross-neutralisation assay.

CHAPTER II

REVIEW OF LITERATURE

The review of literature comprises a comprehensive review of publications on Canine Parvovirus relevant to the objectives taken up for the study.

2.1 Introduction to CPV

'Parvo' means small (Latin), canine parvovirus belongs to genus *Parvovirus* and family *Parvoviridae*. It has an icosahedral symmetry, 25 nm in diameter and nonenveloped with a linear, single stranded DNA genome having size of 5.2 Kb [Cottmore and Tattersall 1982] in length and has two promoters resulting in the expression of three structural (VP1, VP2 and VP3) and two non-structural proteins (NS1 and NS2) through alternate splicing of the viral mRNAs. VP2 (64 kDa) being the major component of the capsid is an NH2-terminally truncated form of VP1 (84 kDa). VP3 which is derived from VP2 by posttranslational proteolytic cleavage is present only in complete (DNA-containing) virions. Empty particles do not contain VP3 protein. Trypsin treatment of full particles cleaves VP2 to VP3 protein. (Nandi *et al* 2007).

It was first isolated in 1978 during a severe pandemic in the dog populations (Appel, Scott & Charmichael 1979). Main clinical signs are haemorrhagic diarrhoea and vomiting. Myocarditis may also occur in puppies infected during the first few days after birth (Berns 1990). Canine parvovirus-2, was named so to differentiate it from CPV-1 (Appel *et al* 1987).

In the family Parvoviridae, genus Parvovirus, canine parvovirus (CPV) and feline pan leukopenia virus (FPV) are classified as host range variants (Siegi *et al* 1985). Diseases associated with FPV, mink enteritis virus (MEV), or raccoon parvovirus (RPV) in cats, minks, or raccoons, respectively, have been known for many years. CPV is closely related to feline panleukopenia virus (FPV), raccoon parvovirus (RPV), and mink enteritis virus (MEV). These viruses are all >98% similar in DNA sequence within the capsid protein gene (Carlson *et al* 1985; Martyn *et al* 1990; Parrish *et al* 1991; Parrish *et al* 1988; Reed *et al* 1988; Rhode 1985). Studies with monoclonal antibodies (MAbs) indicate that the viruses are very similar antigenically as well (Veijalainen 1988).

Along with feline panleukopenia virus (FPV), raccoon parvovirus (RPV), and blue fox parvovirus (BFPV),canine parvovirus 2 comprise the feline parvovirus (FPV) subgroup of the genus *Parvovirus* (Berns *et al* 2000; Ntafis *et al* 2010). CPV-2 and FPV were found to be significant pathogens for domestic dogs and cats as well as for various wild carnivore species. They were grouped along with other viruses such as mink enteritis virus (MEV), raccoon parvovirus (RPV), raccoon dog parvovirus (RDPV) and blue fox parvovirus (BFPV) in the so-called feline parvovirus subgroup (Steinal *et al* 2001)

The DNA sequence was determined and the genome of a canine parvovirus isolate strain (CPV-N) was cloned. The entire genome, including ends, is 5,323 nucleotides in length. Consensus sequences for the 5' donor and 3' acceptor sites as well as promotors and poly (A) addition sites were identified and compared with the available information on related parvoviruses. The genomic organization of CPV-N is similar to that of feline parvovirus (FPV) as there are two major open reading frames (668 and 722 amino acids) in the plus strand (mRNA polarity).The nucleotide and amino acid homologies of the capsid genes between FPV and CPV-N were 99 and 98%, respectively. These results indicated that very few nucleotide or amino acid changes differentiate the antigenic and host range specificity of CPV and FPV (Reed *et al* 1988)

The parvoviruses are the smallest and simplest of the DNA animal viruses. The non-enveloped virion having a diameter of 20 to 26 nm, contains a linear, single-stranded DNA genome of approximately 5 kilobases (kb} encapsidated within a simple icosahedral protein coat composed of three proteins with overlapping amino acid sequences. This family of Parvoviridae (Siegi *et al* 1985) has been divided into three genera: parvoviruses: which can grow in dividing mammalian cells of appropriate species origin; adeno-associated viruses (AAV): which usually require a coinfection with either an adenovirus or a herpesvirus (Bauer and Monreal 1986; Bulier *et al* 1981) for a productive infection in cell culture; densoviruses: which multiply in insects.

Canine parvovirus infection occurs worldwide in domestic dogs and other members of the dog family. Incidences reported to be higher in animal shelters, pet stores, and breeding kennels. CPV can affect dogs at any age although severe

infection is most common in puppies between 6 weeks and 4 months old. All breeds of dogs are susceptible yet the crossbreds are less susceptible in comparison to pure breeds like Rottweilers, Doberman Pinchers, English Springer Spaniels and German Shepherd, the exception to this being Toy Poodles and Cocker Spaniels (Houston *et al* 1996). CPV affects only dogs, and cannot be transmitted to humans or other species. It is studied that if a dog survives the first 4 days, they will usually recover rapidly and become immune to the virus for life. The CPV infection is more severe in young puppies especially those younger than 3 months of age (Appel *et al* 1979; Jacob *et al* 1980). All infected dogs may not necessarily exhibit clinical manifestations but they may shed the virus in feces during the acute phase of enteric fever and show significant rise in the serum antibody titers (Stann *et al* 1984).

There is a specific epitope on CPV which is not found to be present on the other viruses, and these epitopes can distinguish FPV and MEV isolates from CPV (Parrish *et al* 1991; Veijalainen 1988). In addition, CPV isolates hemagglutinate at pH values between 6.0 and 8.0, while FPV isolates hemagglutinate only pH 6.8 (Carmichael *et al* 1980; Moraillon and Moraillon 1982; Parrish 1988). Studies of recombinants between CPV and FPV indicate that sequence differences which affect the antigenic type of CPV and the canine host range are located within the VP1 and VP2 genes, between 59 and 73 map units (m.u.) (Parrish *et al* 1988).

2.2 Antigenic variation

The results of the study by Nandi *et al* in 2010 clearly revealed that CPV variants can be differentiated by sequence analysis of the VP1/VP2 gene. The comparison of VP1/VP2 gene sequences among different CPV variants revealed that the major part of the gene is conserved while changes in specific nucleotide position affect the major antigenic site of the viral capsid that determine the unique variant of CPV (Nandi *et al* 2010)

The differences in nucleotide sequences within the VP-1 lVP-2 gene were used to prepare a network of the phylogenetic relationships between the viruses (Fitch, 1977).

CPV contains the three capsid proteins VP1, VP2' and VP2 (in descending order by size). The mol. wt. of the CPV proteins were calculated to be 82.3 x 103, 67.3 x 103 and 63.5 x 103 for VP1, VP2' and VP2 respectively. One of the

distinguishing characteristics of the parvovirus capsid is that VP2 is formed as a result of a proteolytic cleavage of VP2', which occurs in vivo after the full virus is assembled (Rhode *et al* 1985)

The protein component of the canine parvovirus consists of the more abundant yet smaller - VP2', which can be proteolytically cleaved to VP2 in the mature virus, and the less abundant yet larger - VP 1 (Kongsvik *et al* 1978).

As discussed earlier, the genome is a single-stranded negative-sense DNA, 5.2 kb in length with two promoters which result in the expression of three structural viral proteins (VP1, VP2 and VP3) and two non-structural proteins (NS1 and NS2) (Parrish *et al* 1999). VP2 being 64 kDa in size is an NH2-terminally truncated form of VP1 which is 84 kDa and is the major component of the capsid and plays a major role in virus pathogenicity and host immune response (Mochizuki *et al* 1996). By post-translational proteolytic cleavage, VP3 is derived from VP2 and is present only in complete DNA-containing virions (Reed *et al* 1988).

The mutation rate of the CPV genome has not been determined; however, since parvovirus DNA is replicated by host cell DNA polymerases (Cotmore & Tattersall, 1987), which have low error rates, mutational events affecting the biological properties of parvoviruses occurs very rarely, the rate of variation being 1×10^{-4} to 4×10^{-4} changes/nt/year (Parrish, 1991; Truyen *et al* 1995).

An antigenic variant of CPV was identified in several different countries by using monoclonal antibodies (MAbs) during 1979 and 1980 and the variant was termed CPV type 2a (Parrish *et al* 1985, 1991). The virus underwent a further antigenic change in the mid 1980’s and the new variant was referred to as CPV type 2b (Parrish *et al* 1991). In the current world the antigenic variants of CPV have completely replaced the original type 2 and are variously distributed in canine populations worldwide (Mochizuki *et al* 1993; de Ybanez *et al* 1995; Greenwood *et al* 1996; Truyen *et al* 1996, 2000; Steinel *et al* 1998; Sagazio *et al* 1998; Buonavoglia *et al* 2000; Pereira *et al* 2000).

Following the onset and rapid spread of the antigenic variants 2a and 2b, there has been little evidence of further significant antigenic evolution. Further analysis have demonstrated that during the last 15 years, both CPV types 2a and 2b have retained the antigenic configuration defined originally by Parrish *et al* in1985, 1991).

The antigenic variants differ from the original type in five or six amino acids, while the variant type 2b differs from type 2a only in two positions, Asn-426 to Asp and Ile-555 to Val (Parrish *et al* 1991). Residue 426 is placed in a major antigenic site (epitope A) over the three-fold spike of the capsid and CPV-2b differentiates not only from CPV types 2 and 2a but also from feline parvovirus (FPV) and mink enteristis virus (MEV) by the mutation Asn-426 to Asp. Conversely, residue 555 lies in a minor antigenic site and the mutation of Ile-555 to Val represents a reversion to or a retention of the sequence of the original type 2 (Parrish *et al* 1991; Agbandje *et al* 1995; Strassheim *et al* 1994).

Nucleotide substitutions in CPV-2 continued to be observed. In 1979, a CPV variant (CPV type 2a) emerged that spread worldwide within 1 year due to antigenic drift and replaced the CPV type 2 strains. The study conducted revealed that CPV type 2a contained five substitutions in the capsid sequence compared to CPV type 2, including changes of VP2 residues 87 from Met to Leu, 300 from Ala to Gly, and 305 from Asp to Tyr (Parrish *et al* 1991). CPV type 2a isolates were antigenically different from CPV type 2 and host differences were also observed as it infected and caused disease in cats as well (Truyen *et al* 1996]. In 1984, an antigenic variant of CPV type 2a (CPV type 2b) was recognized and it differed in an antigenic epitope as a result of the substitution of VP2 at residue 426 from Asn to Asp and at residue 555 from Ile to Val (Parrish *et al* 1985). These CPV-2a and CPV-2b are the predominant strains currently circulating in the different dog population, and have completely replaced the original CPV-2 virus worldwide (Parrish *et al* 1991; Truyen *et al* 1996).

Nandi *et al* studied the occurrence of CPV infections in dogs and CPV-2b was found to be the predominant strain in northern India (Nandi *et al* 2010). Furthermore, Chinchkar *et al* studied the epidemiology of CPV infections in dogs in India and found that in central and southern India CPV type 2a isolates were predominant over the CPV-2b variant (Chinchkar *et al* 2006).

The epidemiology of canine parvovirus (CPV) infections in dogs in India was examined using 27 isolates. The VP2 genes of 22 isolates were sequenced, and when the deduced amino acid sequences were compared, the results indicated that the isolates belonged to CPV type 2a except four, which belonged to CPV type 2b.

Comparison of the VP2 gene sequences revealed that the Indian isolates formed separate lineages distinct from the South East Asian isolates (Chinchkar *et al* 2006)

The different antigenic variants of CPV-2 are prevalent in varying proportion in different countries. The prevalence of CPV-2b by various authors in several countries namely Brazil (Pereira *et al* 2000), USA (Parrish *et al* 1988), Japan (Hirasawa *et al* 1996), Switzerland (Truyen *et al* 2000) and South Africa (Steinel *et al* 1998) has been reported. Contrastingly, CPV-2a was found to be the prevalent antigenic type in France, Taiwan and Italy (Chang *et al* 1992; Martella *et al* 2004). However both CPV-2a and CPV-2b have been found to be distributed in equal proportion in Spain and U.K. (Greenwood *et al* 1996). While CPV-2c has been found in Vietnam (Nakamura *et al* 2004), Spain (Decaro *et al* 2006), United Kingdom (Decaro *et al* 2007), South America (Perez *et al* 2007), North America (Kapil *et al* 2007).

Ramadas *et al* isolated CPV-2 for the first time in India in 1982 (Ramadas *et al* 1982). After that, a large number of CPV outbreaks began to report from different parts of India. The incidence of CPV-2 variants in dogs were reported from different states viz. Kerala (Deepa *et al* 2000), Assam (Phukan *et al* 2004), Tamil Nadu (Sanjukta *et al* 2008), Orissa (Banja *et al* 2002), West Bengal (Biswas *et al* 2006), Pondicherry (Panneer *et al* 2008), Haryana (Savy *et al* 2009) and Uttar Pradesh (Nandi *et al* 2010; Murphy *et al* 1999).

Seven strains of canine parvovirus (CPV) were isolated from affected dogs in Japan between 1999 and 2000, and their VP2 genes were genetically analyzed. Comparison of the predicted amino acid sequences of VP2 suggested that three field isolates corresponded to CPV type 2a, while the other four to CPV type 2b (Doke *et al* 2006)

2.4 Vaccination failure

A vaccine is a biological preparation that provides active acquired immunity to a particular infectious disease. A vaccine typically contains an agent that resembles a disease-causing microorganism and is often made from weakened or killed forms of the microbe, its toxins, or one of its surface proteins.

Puppies from immune bitches are protected for the first week of life by maternal antibodies which are acquired via the colostrums. Successful immunization

with most vaccines can be accomplished only in seronegative pups, or in pups with very low antibody titers. Maternal antibodies are acquired during the initial 2–3 days of life and then decline. There is a critical period where maternal antibodies are no longer present in sufficient quantity to confer protection, hence vaccination is required and in case of CPV 90% of the pups from vaccinated populations respond to vaccines at 12 weeks of age (Pratelli *et al* 2000; Truyen 2006).

Vaccination of dogs is generally performed using multivalent vaccines, which contain Canine Distemper Virus (CDV), Canine Parvovirus (CPV), leptospira bacterin and inactivated rabies virus. Monovalent CPV-2 vaccines are also available, containing very high titer virus (10^7 $TCID_{50}$) and widely recommended for initial vaccination of pups. The vaccines are administrated at 6weeks or 8 weeks followed by 12 weeks. The commercial vaccines against CPV available in the Indian market are: parvovirus vaccine (Bio-Core Inc. Company, Kissimmee, Florida) and Nobivac® Puppy DP (distemper parvo) (Intervet, Boxmeer) (Nandi *et al* 2010)

Though maternally derived antibodies play an important role in the protection of the neonate yet are also considered one of the most important causes of immunization failures (Pollock *et al* 1985; Waner *et al* 1996)

Eighty-four of 167 samples (50.3%) were positive for CPV-2 by conventional PCR. Thirty-six of 84 positives samples came from dogs that had received 1–3 doses of various commercial modified live virus CPV vaccines. Seventeen of these dogs were sampled when presenting clinical signs within 10 days of vaccination.

Nandi *et al* in 2010 revealed that to obtain better protection against the field strains of CPV, the incorporation of a specific new variant of CPV-2 (CPV-2a and CPV-2b) in the vaccine is recommended based on the prevalence in the country. (Nandi *et al* 2010)

There have been concerns expressed over the efficacy of canine parvovirus vaccines which are based on the original type 2 strain (Martella *et al* 2005; Truyen 2006). There are studies reporting of gastroenteritis subsequent to vaccination and are related to infection with CPV field strains shortly before or after the vaccine administration (Decaro *et al* 2007). Previously, it had been demonstrated that a type 2 vaccine is able to provide protection against type 2a and 2b field isolates (Greenwood *et al* 1995). Larson *et al* and Spibey *et al* showed that all available vaccines based on

CPV-2 and CPV-2b protect against all known strains of CPV, including CPV-2c strain (Larson *et al* 2008; Spibey *et al* 2008). According to the work done by Nandi in India, most of the vaccines marketed are based on the CPV-2 isolated about 30 years ago [Nandi *et al* 2009; Nandi 2010]. Yet, there are reports of gastroenteritis in vaccinated dogs which is presumed to be due to CPV-2 not being capable enough to provide full protection against the new strains [Savi *et al* 2009].

Killed and Modified Live Vaccine

Initially, a killed CPV vaccine and more recently live and recombinant vaccine had been developed in the search of a product of improved potency. However, because of maternally derived antibodies (MDA), no vaccine has proved to be of high efficacy (McCandlish *et al* 1981). Though live virus vaccines offer a longer duration of immunity than killed vaccines (Schultz 2006), none of the currently available vaccines circumvent maternally derived immunity as effectively as does virulent CPV but it has been studied that MLV-CPV vaccines can overcome a higher concentration of maternally derived antibodies than vaccine containing inactivated virus (Gamoh *et al* 2005).

DNA vaccine

The prokaryotic vector harboring the genes coding for the structural proteins of the canine parvovirus have shown the encouraging results as the dogs immunized with the DNA vaccines withstood the challenge with virulent canine parvovirus. However, the DNA vaccines still is in the experimental stage and not yet licensed to be used in the field condition (Gupta *et al* 2005).

Pratelli *et al* in 2001 revealed that there is evidence to suggest that optimal protection may not be provided if CPV2 vaccines are used, considering that (i) the original CPV2 has disappeared from the canine population worldwide and (ii) the CPV2 vaccine appears to confer a somewhat lower and shorter immunity against the CPV2b biotype. Also it was suggested in 1982 that more effective vaccines that induce longer lived immunity to infection should be sought to control the spread of canine parvovirus (Pollock *et al* 1982) (Pratelli *et al* 2001).

2.5 Diagnosis

The diagnostic tests which were employed earlier for the diagnosis of CPV include HA (Haemagglutination) (Carmichael *et al* 1980), Electron Microscopy (EM)

(Burtonboy *et al* 1979), virus isolation using in MDCK, CRFK or A 72 cell line (Appel *et al* 1979), Enzyme Linked Immunosorbent Assay (ELISA) (Mohan *et al* 1993), Latex Agglutination Test (LAT) by (Appel *et al* 1978), Fluorescent Antibody Test (FAT) and CIE test by (Ramadass *et al* 1982), Virus neutralization test, PCR and RE digestion (Nandi *et al* 2007; Pereira *et al* 2007), real time PCR (Decaro *et al* 2005), loop-mediated isothermal amplification (LAMP) (Cho *et al* 2006), nucleic acid hybridization or dot blot, in situ hybridization, nucleic acid sequencing etc. (Cho *et al* 2004; Nandi *et al* 2010), but it has also been found that they have varying degree of sensitivity and specificity and sometimes yielding false positive cases.

Polymerase chain reaction (PCR) is an efficient and cost-effective molecular tool to copy or amplify small segments of DNA or RNA. PCR combines the principles of complementary nucleic acid hybridization with those of nucleic acid replication that are applied repeatedly through numerous cycles. It results in the exponential production of the specific target DNA/RNA sequences by a factor of 10^7 within a relatively short period.

Several modifications of PCR methods have been developed to enhance the utility of this method in diagnostic settings based on their applications. Some of the common types of PCR are;

1. Real-Time PCR (quantitative PCR or qPCR)
2. Reverse-Transcriptase (RT-PCR)
3. Nested PCR
4. Touchdown PCR

TOUCHDOWN PCR

TD-PCR is a modification of PCR in which the initial annealing temperature is higher than the optimal Tm of the primers and is gradually reduced over subsequent cycles until the Tm temperature or “touchdown temperature” is reached. A gradual lowering of temperature to a more permissive annealing temperature during the course of cycling favors amplification of the desired amplicon.

A touch-down protocol was used and the sensitivity of PCR was as high as ten infectious particles per reaction which corresponds to a titer of about 103 infectious particles per gram of unprocessed feces. This renders the PCR about 10 to

100 fold more sensitive than electron microscopy which was once considered to be a standard method for parvovirus diagnosis. The very rapid and simple sample preparation recommends this PCR assay as an effective technique for routine parvovirus diagnosis (Schunck *et al* 1995)

NESTED PCR

Nested PCR is a modification of PCR designed to increase the sensitivity and specificity of the assay reaction. It involves the use of two primer sets directed against the same target and two successive PCR reactions. The first set of primers is designed to anneal to sequences upstream from the second set of primers, whereas the second set of primers is situated internally or nested with respect to the first set of primers. First set of primers also called "outer primers" amplify a large fragment of the gene which is used as a template in the second round of PCR that targets a smaller region of the amplicon using the second set of primers also known as "inner primers or nested primers."

A polymerase chain reaction (PCR) for the detection of canine parvovirus (CPV) was developed by Hirasawa *et al* in 1994. To increase the sensitibity and specificity of the reaction, the nested PCR with a double-nested primer pair was designed which were selected from the conserved region in the CPV VP1/VP2 gene. The nested PCR was shown to be 100 times more sensitive than the single PCR. The specificity of the reaction was confirmed by restriction enzyme analysis and Southern hybridization. Fecal samples were also examined by the nested PCR. All 10 samples suspected of CPV infection were positive. The number of the genome copy was estimated about 10^{9}–10^{11}/g by the single PCR and 10^{11}–10^{13}/g by the nested PCR in the positive samples. The assay can be completed in 1–1.5 days, and does not need radioisotopes hence rendering the nested PCR to be a sensitive, specific and practical method for the detection of CPV in fecal samples (Hirasawa *et al* 1994)

2.5.1 Real time PCR

Real-time PCR also called quantitative PCR (qPCR) is a variant of standard polymerase chain reaction in which amplification and simultaneous quantitation of a target DNA is done in the same PCR machine, using commercially available fluorescence-detecting thermocyclers. Fluorescent dyes specifically label

DNA of interest and the amount of fluorescence generated is proportional to the quantity of DNA present.

Real time PCR is a rapid, sensitive and reproducible assay for detection and quantitation of canine parvovirus type 2 in the feces of dogs with parvovirus infection. This method is highly specific and sensitive, allowing a precise canine parvovirus type-2 quantitation over range of eight orders of magnitude i.e. from 102 to 109 copies of standard DNA. Fecal samples from parvovirus infected dogs has been analyzed by conventional PCR and real-time PCR. Real-time PCR being more sensitive than conventional PCR detects low viral titers of CPV-2 in infected dogs. By real-time PCR, a wide range of parvovirus particles has been found in the samples from 1.45×106 to 9.45×108 copies/0.01g of feces. (Sin *et al* 2005)

Depending on the available excitation source and detection filters, a variety of fluorescent dyes may be used in qPCR. Two most commonly use real-time PCR methods uses SYBR green (a dye that binds to double-stranded DNA but not to single-stranded DNA, and, when so bound, fluoresces) and TaqMan probes respectively.

PCR using SYBR

SYBR green is a dye which binds to double-stranded DNA but not to single-stranded DNA, and, when so bound, fluoresces. During PCR cycle, as more and more double-stranded product is generated to which SYBR green dye attach and fluoresces, the increasing amount of fluorescent signal is generated. The downside of SYBR green, however, is that it will bind and fluoresce all double-stranded products in the reaction whether they are specific products, nonspecific products, primer dimers, or other amplification artefacts.

The study describes the development of SYBR Green based real-time polymerase chain reaction (real-time PCR) for detection and quantitation of canine parvovirus type 2 (CPV 2) in faecal samples of dogs. A standard curve was plotted using 10-fold serial dilution of standard plasmid DNA and Ct value and the curve was found to be linear over a 10^{-7} dilution. Faecal samples (47) from dogs suspected of CPV 2 infection were analyzed by real-time PCR, haemagglutination (HA) assay and by a conventional PCR and 24, 20 and 22 samples were found positive for CPV 2, respectively. Comparison between the results of revealed that real-time PCR is more

sensitive than HA and conventional PCR and allow the detection of low titers of CPV 2 in infected dogs as the real-time PCR results were expressed as the number of DNA copies of CPV 2 per mg of faecal samples and showed range of 1.0×10^3 to 7.0×10^9 copies of viral DNA per mg of stool samples. The analytical sensitivity of the SYBR Green based real-time PCR was shown to be equivalent to 10 copies (Kumar and Nandi 2010).

TaqMan PCR (5' nuclease assay)

TaqMan PCR uses dye-labelled nucleic-acid probe complementary to an internal segment of the target DNA. This dye-labelled probe anneals to one of the template strands close to and downstream from one of the two PCR primers. The probe is labelled with two fluorescent moieties, the reporter (fluorophore) is attached to the probe's 5' end and the quencher is attached to its 3' end.

When the reporter and the quencher are connected to each other, the quencher reduces the fluorescent signal of the reporter dye as it absorbs the energy via a mechanism known as fluorescence resonance energy transfer (FRET). However, during PCR, Taq polymerase, extending the primer on the probe's target strand, displaces and degrades the annealed probe through the action of its 5' to 3' exonuclease function. The fluorophore is thereby released from its molecular attachment to the quencher and fluoresces.

470 clinical specimens from 346 dogs were examined by PCR and detected MVC-specific gene fragments from four diseased puppies (positive rate, 1.2%) (Mochizuki *et al* 2002)

To combat the problem of antigenic type identification, a de novo multiplex real time PCR was used for identification as well as antigenic typing of CPV. From the study it was concluded that the here developed multiplex real time PCR assay could be used for rapid detection of CPV as well as typing of its three antigenic types.(Kaur *et al* 2016)

To detect CPV-2 DNA, a conventional polymerase chain reaction (PCR) assay using DNA polymerase (Buonavoglia *et al* 2001; Decaro *et al* 2005) with primers Hfor and Hrev was performed. For the characterization of variants and to discriminate between the vaccine and field strains(Decaro *et al*2006), the PCR-positive samples were tested using real-time PCR assays with minor groove binder

(MGB) probes. The MGB probe assays for vaccine and/or field strain discrimination were then applied to the PCR-positive samples which were collected from dogs displaying gastroenteritis within 10 days after vaccination with CPV-2 (Decaro *et al* 2007). 81 samples were characterized as CPV-2a, 1 as CPV-2b, and 2 as CPV-2c, using the real-time PCR with MGB probes. The older dog (GR51/08) had never been vaccinated, the younger one (GR09/09) being fully vaccinated using a classic CPV-2 vaccine. Analysis by MGB-PCR determined that all viruses detected were field rather than vaccine strains (Ntafis *et al* 2010).

Mochizuki *et al* in 1993 and Decaro *et al* in 2005 stated that, PCR is as sensitive as virus isolation and more sensitive than the haemagglutination test for CPV detection from the faecal samples of dogs (Biswas *et al* 2006)

Decaro *et al* employed real-time PCR using the TaqMan assay for the detection of CPV-2 DNA in the sample [Decaro *et al* 2005]. The minor groove binder (MGB) probe technology was applied to obtain rapid and unambiguous identification of the viral type [Decaro *et al* 2006]. The advantage of the real time PCR is that there is no need to analyse the PCR product by agarose gel electrophoresis in fact everything is graphically shown on the monitor of the computer. Also, another advantage is that amount of the DNA present in the sample can be quantitated [Decaro *et al* 2005].

Two real-time PCR assays using minor groove binder (MGB) probes were developed for rapid and unambiguous characterisation of CPV-2 (Decaro *et al* 2005b, Decaro *et al* 2006). Decaro *et al* studied that the MGB probe assays are able to recognise the single nucleotide polymorphisms (SNPs) existing between types 2a/2b (A4062G) and between types 2b/2c (T4064A), which determine the presence at residue 426 of the capsid protein of amino acids Asn, Asp and Glu in types 2a, 2b and 2c, respectively (Parrish *et al* 1991, Buonavoglia *et al* 2001).

In this study, a quantitative TaqMan PCR for detection and quantitation of canine and feline parvoviruses in serum and fecal samples was developed by Streck *et al* The primers were designed for CPV and FPLV following the generation of a standard curve and validation tests were performed using 10-fold serial dilutions of CPV-2 virus in CPV/FPLV-negative feces and CPV/FPLV-negative serum samples. As a result, all canine parvovirus types as well as FPLV were detected. Hence

concluding, the real-time PCR represents an upgraded and useful tool to identify and quantify canine and feline parvoviruses in different sample matrices (Streck *et al* 2013)

Elia *et al* in 2007 developed a TaqMan real-time RT-PCR assay for detection of RNA transcripts produced by replicating CPV-2. A pair of primers and a TaqMan probe targeting the spliced NS2 mRNA were designed and a synthetic DNA fragment was constructed to mimic the spliced NS2 mRNA by PCR-based gene assembly and was used for generation of standard RNAs. The assay was finally applied to determine the mRNA loads in the tissues of dogs naturally infected by CPV-2. mRNA was also detected in a variety of tissues, including the central nervous system (Elia *et al* 2007).

Virus was detected in the feces of 2 liters of pups for 4.5, 6.5, and 46 median days by hemagglutination, virus isolation on cell cultures, and real-time polymerase chain reaction (PCR), respectively. By real-time PCR, the highest viral DNA titers were detected in the feces of both litters at day 10, reaching median values of more than 10^{10} DNA copies/mg of feces (Decaro *et al* 2005).

An *et al* developed a novel peptide nucleic acid (PNA)-based array for use in ante-mortem antigenic typing discrimination in dogs with canine parvovirus (CPV). Synthesis of cyclic benzothiazole-2-sulfonyl PNA monomers that recognized GTA (CPV-2) and TAT (CPV-2a, -2b and -2c) at the nt 913–915 positions, and AAT (CPV-2 and CPV-2a), GAT (CPV-2b), and GAA (CPV-2c) at the nt 1276–1278 positions of the VP2 gene. This PNA array described here was developed from 135 field dog fecal specimens and had 89.8% (62/69) sensitivity and 90.4% (66/73) specificity compared with a real-time PCR using the TaqMan assay, a gold standard method. This CPV PNA array could be used together with MGB probe assays as an attractive novel tool for ante-mortem antigenic typing discrimination (An *et al* 2012)

Ninety-three rectal swab samples taken from the suspected dogs were analyzed by PCR. A fragment of the VP2 gene, was amplified in 41 (44%) of them, resulting CPV positive samples. Sequencing analysis of these PCR products revealed 37 samples (90.2%) belonging to the CPV2c type, whereas four samples (9.8%) were identified as CPV2a, which has not been found since 2008. 24 out of 37 CPV2c samples (65%), carried the mutation Thr440Ala, whereas this mutation was absent in the four CPV2a strains. The results obtained in this work strongly suggested that, in

spite of the geographical proximity of Uruguay and Argentine, wild type CPV strains undergo different evolutive pathways in each country, resulting in the prevalence of different strains in related dog populations. (Calderon *et al* 2015)

The updating of methodologies by incorporating the Minor Groove Binder probe technology, as well as PCR amplification followed by sequencing, allowes a precise identification of emerging CPV variants, therefore becoming important tools for the study of CPV epidemiology (*Decaro et al* 2006a, Decaro *et al* 2005c, Decaro *et al* 2005b).

2.5.2 ELISA

The ELISA test which is rapid, relatively cheap is based on the antigen–antibody reactions with specific MAbs which are fixed on plastic, nitrocellulose membranes, latex or gold particles [Waner *et al* 1996]. The double sandwich ELISA is a rapid, simple, sensitive and suitable test over ELISA for routine diagnostic use for detection of CPV antigen in canine faeces and has become the most common test for parvovirus in puppies (Waner *et al* 2003).

Kumar *et al* developed a polyclonal antibody-based antigen-capture ELISA (AC-ELISA) for the detection of Canine parvovirus (CPV) antigens in faecal samples of dog wherein the assay uses rabbit anti-CPV polyclonal antibody as the capture antibody, guinea pig anti-CPV polyclonal antibody as tracing antibody and anti-guinea pig HRPO conjugate as the detection system. A total of 152 samples (129 faecal samples and 23 cell culture supernatant) were tested both by AC-ELISA and by polymerase chain reaction (PCR). Of the samples tested, 69 samples were found positive and the relative sensitivity, relative specificity and accuracy of AC-ELISA were of 88.4%, 100.0% and 91.4% respectively. The analytical sensitivity of AC-ELISA was estimated to be $10^{2.8}$ $TCID_{50}$/mL. Therefore, AC-ELISA was concluded to be a relatively simple, quick and reliable method for screening large numbers of faecal samples of dogs suspected of CPV infection (Kumar *et al* 2010).

A dot ELISA for the detection of immunoglobulin M (IgM) antibodies to canine parvovirus (cpv) was assessed. The titres of IgM antibodies to cPv in 100 dogs were measured by the Immunocomb ELISA kit and compared with the results derived from the immunofluorescence assay (IFA). There was a strong correlation between the results of the dot ELISA technique and the IFA. The dot ELISA kit was also used

to assess the changes in the levels of immunoglobulin G (IgG) and IgM antibodies to CPV in 10 puppies vaccinated with a polyvalent vaccine. High levels of IgM antibodies to CPV were first detected seven days after they were vaccinated, and after nine days all the pups had high titres of IgG antibodies to CPV(Waner *et al* 2003)

Canine parvovirus (CPV) is a highly infectious and often fatal disease with worldwide distributions, and is an important population management consideration in animal shelters. A point-of-care ELISA test kit is available to detect serum antibodies to CPV and presumptively to predict protective status. The aim of this study was to determine the diagnostic accuracy of the test compared to CPV hemagglutination inhibition titers, using sera collected from dogs housed at animal shelters. The ELISA test was used under both field and laboratory conditions. The test kit yielded accurate results (CPV: sensitivity 92.3%, specificity 93.5) under field conditions. Point-of-care ELISA testing for serum CPV antibody titers was demonstrated to be a useful tool for determining antibody status when making decisions regarding the need for CPV vaccination and also in animal shelters for population management (Litster *et al* 2012).

It has been observed that false negative faecal canine parvovirus (CPV) antigen ELISA results in dogs with CPV infection are common. Proksch *et al* wanted to investigate whether dogs with a false negative faecal CPV antigen ELISA result have milder clinical signs and laboratory changes, a lower faecal virus load, higher faecal and serum CPV antibody titres and a faster recovery than dogs with a positive result. To conduct the study eighty dogs with CPV infection, confirmed by the presence of clinical signs and a positive faecal CPV polymerase chain reaction (PCR), were assigned to two groups according to their faecal antigen ELISA result. The time to recovery, mortality, and the course of the disease were compared between dogs with positive and negative faecal antigen ELISA results. Of the 80 dogs included, 41 (51.3%) had a false negative faecal antigen ELISA result. Laboratory changes, CPV shedding, and outcomes were not associated with faecal antigen ELISA results. It was therefore concluded, low faecal CPV load and antibodies binding to CPV antigen in faeces are likely to be important reasons for false negative faecal antigen ELISA results. Dogs with clinical signs of CPV infection should be retested by faecal PCR (Proksch *et al* 2015).

Several studies have found that interference caused by maternal antibodies is considered a major cause of canine parvovirus (CPV) vaccination failure. In this study, an immunoblot clinic-based enzyme-linked immunosorbent assay (ELISA) method was used to detect CPV antibodies in sera of pregnant bitches and their offspring to study the response of pups to vaccination. The validity of this method was tested in parallel against the standard hemagglutination inhibition (HI) test and the results of the ELISA were also correlated with those of the standard HI method for quantification of CPV antibodies. ELISA helped with the identification of successfully immunized pups, allowing for a more reliable and cost-effective program of vaccination. This study was the first, in which vaccination response to CPV in pups was followed, using a clinic-based ELISA for CPV antibody monitoring (Waner *et al* 1996)

The CPV ELISA detection kit (CSL Ltd., Melbourne Australia) was developed as a rapid field test for the detection of canine parvovirus (CPV) antigen in canine faecal samples. The CPV ELISA was compared to the haemagglutination assay (HA) test using electron microscopy (EM) and/or virus isolation (VI) to confirm infection. The CPV ELISA had a sensitivity of 87% and a specificity of 100%. Therefore Drane *et al* concluded that CPV ELISA requires no specialised equipment, is simple to perform and provides a visible result in less than 15 minutes (Drane *et al* 1994).

A double antibody sandwich (DAS) ELISA for the detection of CPV in dog faeces was compared with the haemagglutination (HA) test. It proved to be even more specific, sensitive and easier to perform than the HA assay. An indirect ELISA and a competitive ELISA for the detection of CPV-specific antibodies in dog sera were compared with the haemagglutination inhibition (HI) test and both ELISA systems were proved to be specific and easy-to-use methods for the detection of CPV-specific antibodies. The indirect ELISA, specially, proved to be more sensitive and specific than the HI test (Rimmelzwaan *et al* 1990).

2.5.3 Serum virus neutralization (SVN)

A neutralizing antibody defends a cell from an antigen or infectious body by inhibiting or neutralizing any biological effect. The antibody response is crucial for preventing many viral infections and may also contribute to the resolution of an

infection. When a vertebrate is infected with a virus, antibodies are produced against many epitopes of multiple virus proteins. A subset of these antibodies can block viral infection by a process called neutralization. This usually involves the formation of a virus-antibody complex.

CROSS NEUTRALISATION TEST

Serum Neutralisation Test (SNT)

The serum virus neutralization (SVN) assay is a serological test to detect the presence and magnitude of functional systemic antibodies that prevent infectivity of a virus. Conventional SVN methods performed in vitro are based on inhibition of virus infectivity in cell culture in the presence of neutralizing antibodies. Titer determination may be based on the presence or the absence of cytopathic effect or the evidence of viral infection using an immunoreactive technique. The SVN assay is relatively inexpensive using standard laboratory equipment although it requires cell culture, more time and labor, and technical skill to conduct the assay compared to other serological methods. The SVN test is useful to evaluate the level of serological cross-reactivity between vaccine antisera and variant viruses subtypes that may correlate with cross-protection in the host.

Virus Neutralisation Test (VNT)

Viral neutralization tests are used to either identify an unknown virus using known reference antisera/monoclonal antibody or measure virus neutralizing antibody levels in serum samples against a known infectious virus. Specific antibody levels in an animal serum indicate previous exposure to a particular virus and often may give an indication of the susceptibility to a virus.

To test the amount of neutralizing activity, particularly against the heterologous types, studies with antisera which were raised against the original CPV-2 and its variants have been performed. These studies have revealed substantial difference in the neutralization titers and have suggested that the hemagglutination (HA)-inhibiting antibodies do not correlate well with the neutralizing antibodies and may incorrectly estimate the protective immunity against the antigenic variants in pups with passively acquired antibodies against the original type of CPV (Pratelli *et al* 2001; Truyen, 2006)

Neutralizing antigenic relationships between the isolates and the reference parvovirus strains were examined by using polyclonal immune sera against MVC (GA3, HM-6, and 97-114 strains) and CPV-2 (p85 strain). Reaction mixtures contained 100µl of each serum dilution and 100µl of virus fluid containing approximately 100 50% tissue culture infective doses in a 96-well flat-bottom plate (Iwaki Glass) and were incubated at 37°C for 1 h, and 50 µl of each mixture was inoculated into a Lab-Tek II Chamber Slide well (Nalge Nunc International, Naperville, III.) containing 4×10^4 MDCK cells in 0.4 ml of Eagle's minimal essential medium with 7.5% fetal bovine serum. Two chambers were used for each serum dilution. Then the slides were incubated at 37°C for 7 days and further stained by IFA. Therefore, the titer was defined as the reciprocal of the highest serum dilution at which virus growth was completely inhibited.(Mochizuki *et al*2002)

The earliest CPV positive sera reported was in Greece during 1974, and the reactivities of the of 28 dogs examined from that year were confirmed by virus neutralization assays (Koptopoulos *et al* 1986). Several studies of the antigenic relationships between FPV or MEV and CPV were performed using polyclonal sera and revealed that the viruses all gave similar titers in neutralization or hemagglutination inhibition tests, although indication of higher titers in the homologous than in the heterologous reactions were observed (Flower *et al* 1980; Lenghaus and Studdert, 1980; Parrish *et al* 1982b; Tratschin *et al* 1982).

To determine cross-reactivity, Pratelli *et al* devised two CPV vaccines, CPV2 vaccine and a CPV2b vaccine. i) CPV2 vaccine- A modified live CPV2 vaccine (17/80 ISS strain) (Buonavoglia *et al* 1983) with a titer of 105.50 tissue culture infectious doses (TCID50)/ml was used. Virus cultivation on the canine A-72 cell line grown in Dulbecco minimal essential medium (DMEM) supplemented with 10% fetal calf serum. (ii) CPV2b vaccine. A modified live CPV2b vaccine (29/97-40 strain) (Buonavoglia *et al* 1998) with a titer of 104.50 TCID50 was used. Virus cultivation on the Crandell feline kidney (CrFK) cell line grown in DMEM supplemented with 10% fetal calf serum (Pratelli *et al* 2001)

Pratelli *et al* performed the virus titration test in microtiter plates. Tenfold dilutions of each virus were prepared in quadruplicate in DMEM and mixed with 50 ml of a suspension containing 200,000 A-72 cells for CPV2 vaccine and 200,000

CrFK cells for CPV2b vaccine. These plates were incubated at 37°C for 5 days in a humidified CO2 atmosphere further they were frozen and thawed three times, and the supernatant of each well was tested for CPV hemagglutination (HA) activity using 1% pig erythrocytes. Using the Karber formula fifty percent end points were calculated (Pratelli *et al* 2001)

For the calculation of neutralizing antibody titers serial twofold dilutions (starting from 1:10) of each serum in DMEM were mixed with 50 ml of a virus suspension containing 100 to 300 TCID50 of either CPV2 (17/80 ISS strain) or CPV2b (29/97-40 strain). After 1 hr of incubation at room temperature, 100 ml of a suspension containing 200,000 A-72 cells for CPV2 and 200,000 CrFK cells for CPV2b was added to each well. For the detection of virus using the HA test the plates were further incubated at 37°C in a humidified CO2 atmosphere for 5 days. Neutralizing antibody titers were calculated as the reciprocal of the highest serum dilution that completely neutralized the virus (absence of HA activity) (Pratelli *et al* 2001). The results obtained by Pratelli *et al* in 2001 by conducting serological test revealed that pups inoculated with CPV2 vaccine had significantly higher neutralising antibody titers to the homologous virus (CPV2) than to the heterologous virus (CPV2b). In contrast, pups inoculated with CPV2b vaccine had similar neutralising antibody titers to both viruses.

The prevalence of canine parvovirus (CPV) variants in dog was investigated in Korea in a total of 51 fecal samples submitted over a 2-year period (2005–2007). The CPV VP2 gene was amplified and sequenced from the fecal samples indicating that of the 51 samples, 49 samples belong to the CPV-2a family, 1 to CPV-2b, and the remaining 1 to CPV-2a variant. The VP2 gene of 20 isolates was sequenced and phylogenetic analysis was conducted. Their findings showed that CPV-2a was the predominant type and CPV-2b and CPV-2a variant also existed in Korea. The neutralization (Nt) test was used and it was found that the animals inoculated with CPV-2 developed low antibody titers against the CPV-2 variants in laboratory animal and it was identified as well (Kang *et al* 2008).

The A72 canine fibroma-derived cell line (approximately passage 150) (Binn *et al* 1980) and the NLFK feline cell line (derived from the Crandell feline kidney cell line [CRFK] [Crandel *et al* 1973]) were grown (Parrish and Carmichael 1986) in

RPMI 1640 medium with 20% fetal bovine serum (CT 45-S), Dulbecco's minimal essential medium with 10% fetal bovine serum or a 50% mixture of McCoy's 5A and Leibowitz L15 media with 5% fetal bovine serum (Truyen and Parrish 1992)

The antigenic relationships among the original canine parvovirus type 2 (CPV-2) and the variants CPV-2a, -2b, and -2c were evaluated. Cross-antigenic evaluation revealed clear differences among the CPV variants, which were appreciable by serum neutralization (SN). Antigenic differences were found mostly between the original CPV-2 and the variants, but they were also observed among the variants CPV-2a, -2b, and -2c. (Cavalli *et al* 2008)

Virus preparation in rabbits

According to Casal *et al* since there is a good correlation in the antipeptide response in both rabbits and dogs, regarding antibody titer and specificity at the amino acid level (Langeveld *et al* 1994), it is expected that the results obtained in rabbits will be valid also in dogs. Therefore rabbits are used to raise hyperimmune sera aginst CPV and this could help us improve the vaccine status regarding CPV (Casal *et al* 1995).

A mixture of two nondialyzed KLH-peptide conjugates i.e. peptides iL15 and 7L15, 1 mg each was injected into dogs and rabbits. For immunization of rabbits, the immunization cocktail (total volume, 2 ml) consisted of the same mixture of KLHconjugated peptides iL15 and 7L15 as used in the dogs, adsorbed to aluminium hydroxide plus QuilA, and administered by subcutaneous and intramuscular routes. The animals further received a second injection of the same cocktail on day 42 (Langeveld *et al* 1994).

For the mapping studies, Langeveld *et al* used anti-CPV antibody samples from four different animal species were used: naturally infected dogs (six animals), a guinea pig and two rabbits immunized with CPV, and mice immunized (Langeveld *et al* 1993).

Coupled peptides emulsified with Freund’s complete or incomplete adjuvant for first or second injection, respectively, were used to immunize New Zealand White rabbits (intramuscularly and subcutaneously). For rabbit immunizations, each dose contained 1 mg of keyhole limpet hemocyanin protein with the amount of peptide

coupled varying between 60 and 600 nmol/mg of keyhole limpet hemocyanin (Casal *et al* 1995)

Vihinen-Ranta produced antibodies in rabbits against an 18-amino acid peptide (peptide 1, NSLPQSEGATNFGDIGVP) of capsid protein VP2/residues 292–309 of canine parvovirus (CPV) or against an 18-amino acid peptide (peptide 2, GKRNTVLFHGPASTKGKS) of nonstructural protein NS1/residues 391–409 of CPV identified, in immunofluorescence analysis, viral antigens in canine A-72 cells infected with CPV. The peptide antibodies was found to be convenient tools in diagnosis of infections caused by CPV or closely related viruses affecting cats, minks, blue foxes and raccoon dogs (Vihinen-Ranta *et al* 1996).

The objective of the study was to determine serum canine parvovirus (CPV) antibody titers in healthy dogs which were brought to a veterinary hospital for revaccination. Serum antibody titers were measured by means of hemagglutination inhibition (CPV titers) and serum neutralization at the time 122 dogs were brought to a veterinary hospital for revaccination. The dogs had been vaccinated between 271 and 1,665 days previously. To determine whether these factors were associated with antibody titers, these dogs were grouped by age, breed, sex, and weight. Serum CPV titers > or = 1:80 were considered protective. It was concluded that breed, sex, and weight were not significantly associated with serum CPV titers although age was significantly associated with CPV titer, with younger dogs having higher titers. Thirty-three of 122 dogs had a less-than-protective CPV titer. Results also suggested that, on the basis of serum antibody titers, the current practice of annual revaccination of dogs against CPV infection should be maintained (McCaw *et al* 1998).

A study using four CPV-2 strains was conducted. Strain 17/80 ISS, with a titer of 3.2×10^5 50% tissue culture infectious doses ($TCID_{50}$/50 µl), was used to represent the original CPV-2. Strain 192/98 (3.2×10^3 $TCID_{50}$/50 µl) was used to represent CPV-2a variant. Strain 29/97 (3.2×10^4 $TCID_{50}$/50 µl) and strain 136/00 (3.2×10^3 $TCID_{50}$/50 µl) were used to represent CPV variants 2b and 2c, respectively. Titration of the viral strains was performed in microtiter plates. Tenfold virus dilutions were prepared in DMEM in quadruplicates and were added to wells with 2×10^4 A-72 cells/per well. The plates were incubated at 37°C for 4 days in a

CO_2 atmosphere, then they were frozen and thawed three times, and the undiluted cryolysate of each well was tested by HA using 1% pig erythrocytes. Using the Karber method, the virus titer was considered the end point dilution showing HA activity in 50% of the wells. (Cavalli, 2008).

CHAPTER III

MATERIALS AND METHODS

3.1 To identify the antigenic types of CPV using real time PCR

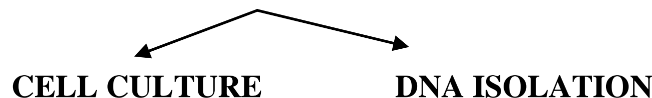
3.1.1 Real time PCR typing of CPV

A real time PCR was conducted on a total of 178 samples (rectal swabs) available in the Department of Veterinary Microbiology, GADVASU, Ludhiana, Punjab. The rectal swabs from suspected dogs available in the department of Veterinary Microbiology were collected from different states of India i.e. Punjab, Assam, J&K, Delhi and Chandigarh. The DNA was extracted from the samples using Phenol-Chloroform extraction method (Sambrook and Russel, 2001). The purity of the DNA was checked using Nanodrop (ThermoFisher, USA) at the absorbance ratio of 260/280. The extracted DNA was subjected to Real time PCR typing for three strains of CPV viz. CPV 2, CPV 2a and CPV 2b.

3.1.2 DNA isolation and preparation of virus inoculums for cell culture

I. 4 ml of sample was centrifuged at 3000 rpm for 15 min at 4°C

II. The supernatant was equally divided in two eppendorf tubes (2ml each)



CELL CULTURE **DNA ISOLATION**

CELL CULTURE (Preparation of virus inoculums of five samples):

a. Collect 600µl of supernatant in an eppendorf tube
b. To the supernatant add 10µl of antibiotic and antimycotic solution (Hi-Media)
c. Incubate at 37°C for 30 min
d. Store at -20°C

Composition of antibiotic and antimycotic solution

Antibiotic-Antimycotic solution 100x liq Endotoxin

- 10000 U Penicillin
- 10mg Stretomycin
- 25µg Ampicillin
- 8% Normal saline

DNA ISOLATION (From samples and vaccines, Nobivac DHPPi):

a. Centrifuge 2ml sample at 3000 rpm for 5 min at 4°C
b. Collect supernatant in an eppendorf tube (600µl)
c. Add 20µl proteinase K (20mg/ml)
d. Add 20µl of 10% SDS (Sodium Dodecyl Sulphate)
e. Vortex the tubes
f. Incubate at 56°C for 2-3 hrs at water bath
g. Add 600µl of phenol-chloroform-isoamyl alcohol (PCI) mixture which is in the ratio of 25:24:1
h. Vortex for 15 sec
i. Centrifuge at 13000 rpm for 15 min at 4°C
j. Collect the aqueous layer of supernatant in a separate eppendorf tube
k. Add 600µl of chloroform and isoamyl alcohol mixture which is in the ratio of 24:1
l. Gently mix
m. Centrifuge at 13000 rpm for 15 min at 4°C
n. Collect supernatant in another eppendorf and add $1/10^{th}$ volume of sodium acetate (3M, pH 5.2)
o. Add 600ul of isopropanol to the mixture and mix gently
p. Keep at -20°C overnight
q. Centrifuge 12000 rpm for 10 min at 4°C
r. Collect pellet and discard supernatant
s. Wash the pellet twice with 70% ethanol
t. Dry it in incubator at 37°C
u. Suspend the pellet in 50µl NFW
v. Store at -20°C till further use

3.1.4 Procedure for preparing Real time PCR mix:

The extracted DNA from the rectal swabs samples was subjected to Real Time PCR for antigenic typing using three assays for CPV 2, CPV 2a and CPV 2b. The 20µl of Real Time PCR reaction mixture was prepared by adding 10µl of 2X

Taqman®Universal Master Mix II with UNG (Applied Biosystems), 1µl of the 20X Taqman® assay (for the individual antigenic type), 2µl of the DNA template and 7µl of Nuclease Free Water (NFW) (Table 2). The PCR reaction was carried out in CFX™ 96 Real-Time System (BioRad, USA) with the thermal condition of UNG incubation at 50°C for 2 minutes, polymerase activation at 95°C and 40 cycles of denaturation at 95°C for 15 seconds and annealing at variable temperatures and time depending on the antigenic type detected. For detection of CPV-2 the annealing was done at 52°C for 30 seconds; for CPV-2a the annealing was done at 61°C for 45 seconds and for CPV-2b annealing was carried out at 57°C for 45 seconds. A rectal swab from a healthy dog was used as a negative control and vaccine (Nobivac DHPPI) DNA was used as a positive control (Table 3).

Reconstitution of Primer probe Assay

Table 1: Real Time PCR assays for three CPV types

Antigenic Type	Taqman Assay	Sequence	References	Position in genome
CPV-2	F	5'-AAACAGGAATTAACTATACTAATAT ATTTA-3'	Decaro *et al* 2005	-
	R	5'-AAATTTGACCATTTGGATAAACT-3'		-
	Probe	5'-/6-FAM/TGGTCCTTT/ZEN/AACTGCATTAAAT AATGTACC/IowaBlack/3'		-
CPV-2a	F	5'-TGACCAAGGAGAACCAACTAAC-3'	Decaro *et al* 2006	847-866
	R	5'-TGATCTGCTGGCGAGAAATATAA-3'		1013-993
	Probe	5'/6-FAM/ACGCTGCTT/ZEN/ATCTTC GCTCTGGT/IowaBlack/-3'		867-896
CPV-2b	F	5'-ACAGGAAGATATCCAGAAGGAGA-3'	Kaur *et al* 2016	1216-1238
	R	5'-TGACCATTTGGATAAACTGGTGG-3'		1403-1381
	Probe	5'-/HEX/TATTAACTT/ZEN/TAACCTTCCTG TAACAGATGA-/IowaBlack/-3'		1251-1280

Table 2: Real Time PCR Master Mix

Components	Quantity
2X Taqman®Universal MM II (with UNG)	10µl
20X Taqman® assay	1µl
DNA Template	2µl
NFW	7µl

Table 3: Thermal conditions for each CPV strain

CPV Strain	UNG hold	Initial Denaturation	Denaturation	Annealing
CPV 2	50°c ; 2min	95°c ; 3min	95°c ; 15sec	52°c ; 30sec
CPV 2a	50°c ; 2min	95°c ; 3min	95°c ; 15sec	61°c ; 45 sec
CPV 2b	50°c ; 2min	95°c ; 3min	95°c ; 15sec	57°c ; 45sec

3.1.4 Determination of Endpoint

The samples were considered positive or negative in the Real-Time PCR depending upon the fluorescence of a particular wavelength emitted by the respective fluorophore attached to the particular probe for the three antigenic types (CPV-2, CPV-2a and CPV-2b) of CPV. Depending upon the highest and lowest relative fluorescence unit (RFU) value, the cut off value or end point was calculated by using CFX Manager Version 3.1.

Table 4: Details of the samples subjected to Real-time PCR for typing of CPV

Sample number	Vaccination status	Age	Sex	Breed
A1	No	3 months	M	Labrador
A2	No	2-3 months	F	Local
A3	No	2 months 15 days	M	German Spitz
A4	No	3 months	F	Golden Retriever
A5	No	2 months 15 days	M	Labrador
A6	Yes	3 months 15 days	M	Local
A7	No	2 months	M	Local
A8	No	2 months	F	Local

Sample number	Vaccination status	Age	Sex	Breed
A9	No	11 months	F	Labrador
A10	Yes	6 months	F	Cocker Spaniel
A11	No	3 months	M	Local
A12	Yes	7 months	F	German Spitz
A13	No	12 months		Local
A14	No	1 months 13 days	M	Labrador
A15	No	2 months	F	German Spitz
A16	Yes	4 months	M	Labrador
A17	No	1 month 10 days	F	Labrador
A18	No	3 months	M	Golden Retriever
A19	Yes	7 months	F	Local
A20	No	3 months	M	Local
A21	Yes	11 months	F	Labrador
A22	No	4 months	M	German Shephard
A23	Yes	2 months	M	German Spitz
A24	Yes	6 months	F	Local
A25	No	3 months	M	German Shephard
A26	No	2 months	F	Labrador
A27	No	2 months	M	Golden Retriever
A28	Yes	2 months	M	German Spitz
A29	Yes	60 months	M	Golden Retriever
A30	No	3 months	F	Local
A31	No	2 months	M	Cross
A32	No	2 months 15 days	M	Local
A33	No	20 days	M	Labrador
A34	Yes	9 months	F	Labrador
A35	No	2 months 15days	M	Local

Sample number	Vaccination status	Age	Sex	Breed
A36	No	1 month 15 days	F	Golden retriever
G1	Yes	5 months	M	Rottweiler
G2	No	2 months	M	German Spitz
G3	No	2 months 15 days	F	Local
G4	No	4 months	M	Local
G5	No	1 month	M	Rottweiler
G6	No	1 month 25 days	M	German Shepherd
G7	No	25 days	M	Labrador
G8	No	1 month 12 days	F	Labrador
G9	No	1 month 8 days	M	Local
G10	No	1 month 15 days	M	German Spitz
G11	No	1 month 10 days	M	German Shephard
G12	No	1 month	M	Pit Bull
G13	No	2 months	M	German Shephard
G14	No	1 month 10days	F	Dachshund
G15	Yes	1 month 23 days	M	Pit Bull
G16	Yes	9 months	F	German Spitz
G17	No	2 months	F	Labrador
G18	No	2.5 months	M	German Spitz
G19	No	1 month 10 days	M	Labrador
G20	No	36 months	F	Local
G21	No	2 months 15 days	M	German Spitz
G22	Yes	3 months	M	Rottweiler
G23	Yes	4 months		Local
G24	No	3 months	M	German Spitz
G25	Yes	4 months	F	Labrador
G26	Yes	48 months	M	Labrador
G27	No	2 months 15 days	F	Pit bull
G28	No	2 months	F	Local

Sample number	Vaccination status	Age	Sex	Breed
G29	No	1 month	M	German Spitz
G30	No	3 months	F	Labrador
G31	No	3 months	M	Labrador
G32	No	11 months	M	German Spitz
G33	No	2 months	M	Local
G34	No	3 months	F	Labrador
G35	No	2 months	M	Pit bull
G36	No	2 months	M	German Shephard
G37	No	3 months	F	Local
G38	Yes	2 months	F	Labrador
G39	No	1 month 10 days	M	Labrador
G40	Yes	2 months 15 days	M	Rottweiler
G41	No	3 months	F	Local
G42	No	2 months	M	Pit bull
G43	No	3 months	M	Labrador
G44	No	6 months	F	Golden Retriever
G45	Yes	2 months 15 days	M	Rottweiler
G46	No	2 months	M	Labrador
G47	No	1 month 15 days	F	German spitz
G48	No	2 months	M	German Shephard
G49	No	3 months	F	Local
G50	No	1 month 10 days	M	Labrador
G51	No	2 months	M	German Shpehard
G52	Yes	3 months	M	American bulldog
G53	Yes	3 months	M	American bulldog
G54	No	2 months	M	German shepherd
G55	No	2 months 15days	F	Local

Sample number	Vaccination status	Age	Sex	Breed
G56	Yes	4 months 15 days	M	Labrador
G57	No	2 months	F	German shepherd
G58	No	3 months	M	Labrador
G59	No	2 months	M	Labrador
G60	No	1 month 15 days	F	German Shephard
G61	No	2 months	M	Labrador
G62	No	3 months	M	Labrador
G63	No	2 months	M	Golden retriever
G64	No	2 months and half month	F	Labrador
P1	No	3 months	F	Mongreal
P2	Yes	3 months	M	Labrador
P3	No	1.5 months	M	Labrador
P4	Yes	4 months	M	Indian Bully
P5	Yes	6 months	M	Pit Bull
P6	Yes	3 months	M	Labrador
P7	No	6 months	M	Mongreal
P8	Yes	1.5 months	M	Pomeranian
P9	No	1 months	F	Pomeranian
P10	No	1 month	F	American Bully
P11	No	1.5 months	M	Labrador
P12	No	4 months	M	Mongreal
P13	Yes	6 months	M	Pomeranian
P14	No	6 months	M	Mongreal
P15	No	2.5 months	M	Mongreal
P16	No	1.5 months	F	GSD
P17	Yes	6 months	F	Pitbull
P18	No	2 weeks	M	Mongreal
P19	No	6 months	F	Pomeranian

Sample number	Vaccination status	Age	Sex	Breed
P20	Yes	4 years	F	Pomeranian
P21	Yes	6 months	M	Pomeranian
P22	No	2 months	M	Mongreal
P23	No	3 months	M	Mongreal
P24	Yes	3 years	M	Dalmatian
P25	No	45 days7 years	F	Pomeranian
P26	Yes	2 months	M	Chihuahua
P27	Yes	8 months	M	GSD
P28	Yes	42 days	F	Cocker Spaniel
P29	No	3 months	M	Beagle
P30	Yes	3 months	F	Chow-Chow
P31	No	4 months	M	Mongreal
P32	No	4 months	M	Mongreal
P33	No	3 months	M	Mongreal
P34	No	5 years	M	Mongreal
P35	Yes	2 months	M	Indian Bully
P36	No	4 months	M	Mongreal
P37	No	4 months	M	Landseer
P38	No	45 days	M	Labrador
P39	No	3 months	M	Pomeranian
P40	Yes	45 days	M	Labrador
P41	No	4 years	M	GSD
P42	No	2 months	M	Dachshund
P43	No	2.5 months	M	Labrador
P44	No	5 months	M	Pomeranian
P45	Yes	2 months	F	Pitbull
P46	No	2 months	M	Pomeranian
P47	Yes	3 months	M	Mongreal
P48	Yes	6.5 months	M	Labrador
P49	No	1.5 months	F	Beagle
P50	No	4 months	F	Labrador

Sample number	Vaccination status	Age	Sex	Breed
P51	No	3 months	M	Dachshund
P52	No	1.5 months	M	Labrador
P53	No	4 months	F	Labrador
P54	No	3 months	F	Pomeranian
P55	No	1.5 months	M	GSD
D1	No	1.5 months	F	Mongreal
D2	No	1.5 months	M	Mongreal
D3	No	1.5 months	F	Mongreal
D4	No	1 month	M	Mongreal
D5	No	2 months	M	Mongreal
D6	No	2 months	M	Mongreal
D7	No	2 months	M	Mongreal
D8	No	1.5 months	F	Mongreal
D9	No	2 months	M	Mongreal
D10	No	2 months	M	Mongreal
D11	No	2 months	M	Mongreal
J1	No	4 months	F	Mongreal
J2	No	9 months	F	Rottweiler
J3	No	1.5 months	F	Mongreal
J4	No	3.5 months	M	Mongreal
J5	No	7 months	M	Mongreal
J6	Yes	3 months	M	Mongreal
C1	No	4 months	F	Mongreal
C2	Yes	3.5 months	F	Doberman
C3	No	2.5 months	M	Labrador
C4	No	1.5 months	M	Beagle
C5	Yes	3 months	M	Labrador
C6	Yes	4 months	F	Cocker spaniel

3.2 To identify antigenic relationship of canine parvovirus types using *in-vitro* cross- neutralisation assay

3.2 CELL CULTURE (Production of virus in bulk)

Growth media preparation: Dulbecco's Modified Eagle Medium (DMEM) (GIBCO) was used for cell culture.

DMEM composition:

- 1g glucose/L
- L-Glutamine
- Na-Pyruvate (110mg/L)
- Na Bicarbonate (3.7g/L)

Composition of GM-DMEM (10% FBS)

Stock DMEM	90ml
FBS (HIMEDIA)	10ml
Antibiotic and antimycotic solution	0.1ml
Total	100ml

The growth media was passed through a filter of size 0.22µm and incubated at 37°C for 2 days for sterility check.

MAINTAINANCE MEDIA (MM-DMEM) with 2% FBS

DMEM	98ml
FBS	2ml
Antibiotic and antimycotic solution	0.1ml
Total	100ml

The growth media was passed through a filter of size 0.22µm and incubated at 37°C for 2 days for sterility check.

ANTIBIOTIC and ANTIMYCOTIC solution (100x) (HIMEDIA)

- 10000mg Penicillin
- 10mg Streptomycin
- 25µg Ampicillin

- 8% Normal Saline

  Trypsin Solution (HIMEDIA)

  Cryopreservation Media (HIMEDIA)

3.2.2 Culture of MDCK (Madin Darby Canine Kidney) cell line

MDCK cell line (Passage no 71 (P 71) was procured from the School of Animal Biotechnology, GADVASU, Ludhiana. One vial of MDCK cell line was taken and transferred immediately to a beaker with lukewarm water at 37°C. After it was thawed, 7.0 ml of growth media was transferred into a 15ml centrifuge tube. Contents of MDCK vial were transferred into the centrifuge tube containing media. The tube was centrifuged at 10000 rpm for 10 min. The supernatant was discarded while the pellet was retained. To the pellet again 5.0ml of growth media was added and centrifuged at 10000rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in 2.0ml of GM-DMEM and transferred to 25cm^2 cell culture flask (GREINER BIO-ONE). The GM-DMEM was added to the cell culture flask to make final volume 5.0ml. The flask was incubated at 37°C with 5% CO2 and observed daily under inverted microscope (NIKON ECLIPSE TS100) for the formation of monolayer.

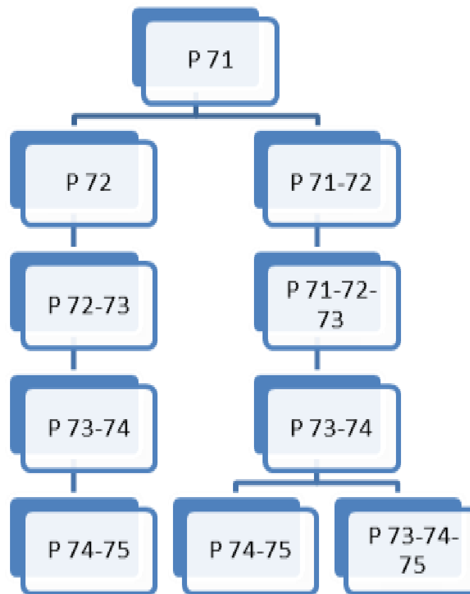
On DAY 1, the cells under inverted microscope appeared shining, globular in shape and. The cell culture plate was incubated at 37°C with 5% CO2 until the monolayer was formed.

DAY 2, adherence began and the adhered cells were epithelioid in shape.

DAY 3, all cells are adhered and monolayer was complete.

3.2.3 CELL PASSAGING/ Subculture of MDCK cell line

Subculture of MDCK cell line was carried out when the monolayer in the cell culture flask was 80 to 90% complete. After the monolayer was formed, growth media was discarded and to the culture flask was added 0.1 ml of trypsin solution. The MDCK cells were incubated with trypsin solution till the cells were completely detatched from the surface of the cell culture flask. Then 5.0ml of maintenance media was added and the cells were split in two 25cm^2 cell culture flasks in the split ratio of 1:2. The flasks were incubated at 37°C, 5% CO_2. The complete monolayer was observed in the flasks on DAY 3. Therefore, the passages were done as:



3.2.4 Cryopreservation OF MDCK cell line

The MDCK cells were cryopreserved at **P 71-72-73 and P 72-73.** The cell-culture flasks had complete monolayer of cells. To each flask 0.1ml of trypsin solution was added and incubated for 5 minutes at 37°C for detachment of cells. The DMEM media was added and the contents were transferred into the centrifuge tubes and centrifuged at 5000 rpm for 5 min. The supernatant was discarded while the pellet was retained. To the pellet 1.0ml of cryopreservation media was added drop wise and the contents transferred to the cryovial. The cryovials were initially kept at -20°C for 2 hours and then kept at -80°C for 2 to 3 hours and then transferred to the neck of liquid nitrogen cylinder for overnight and finally dipped into the liquid nitrogen with proper labelling.

3.2.5 Virus inoculation in MDCK cells

The P 73-74 vial of MDCK cells was further passaged to P 74-75 and the contents were transferred into a 75cm^2 culture flask and were kept for incubation at 37°C, 5% CO_2 for the formation of monolayer of cells and also to 6-well culture plate (1ml/well). When monolayer was complete in wells of 6-well cell culture plate, the virus inoculums of five samples was inoculated in the plate. The processed samples included were G28, G30, A35, P4 and P5.

For inoculation of processed samples, first GM-DMEM was discarded from the wells of 6-well cell culture plate and 500µl of inoculums was added. One well was kept for negative indicator in which PBS was added. The plate was incubated for 1

and ½ hr at 37°C. After incubation the inoculums was discarded from the wells and 1.0 ml of maintenance media (MM-DMEM) was added in all the wells. The plate was further incubated at 37°C with 5% CO_2 and observed daily for cytopathic effects (CPE).

3.2.6 Freezing and thawing/ Harvesting of virus from MDCK cells

For harvesting the virus from the inoculated cell line and passaging it further for production of virus in bulk, the cell-culture plate was freezed at -20°C and then thawed after 12-14 hrs. This process of alternate freezing and thawing was repeated for 3 times. After the third round of freezing and thawing, the cell culture supernatant was collected in eppendorf tubes for each sample and centrifuged at 5000rpm for 5 min and the supernatant was collected and stored at -20°C for further passaging for production of virus in bulk. One sample showing good CPE was selected for virus production in bulk according to the following flow chart.

3.2.7 Production of CPV-2b in bulk

The CPV 2b isolate was procured from Department of Veterinary Biotechnology, College of Veterinary Science, PVNR TVU, Rajendranagar, Hyderabad. It was also produced in bulk in the MDCK cell line to be used in cross neutralization test as indicated in the flow chart in the same way as the local isolate was produced in bulk.

3.2.8 Confirmation by PCR and Nested PCR

The cell culture supernatant was confirmed by PCR and Nested PCR for Canine Parvovirus. The DNA was extracted from cell culture supernatant using Phenol-chloroform method (Sambrook and Russell, 2001) and subjected to PCR and Nested PCR for confirmation of virus.

Procedure for PCR

PCR reaction mixture was made for the confirmation of virus by adding 5µl of 10X PCR buffer (with 15mM $MgCl_2$) (TaKaRa), 1.0µl of forward and reverse primer each (20pm/l each), 1.0µl of dNTP’s (10mM each) (TaKaRa), 0.2µl of Taq polymerase (5 units/l) (QIAGEN)i, 15.0µl of the template DNA i.e. the cell culture supernatant DNA to be confirmed and 26.8µl of Nuclease free water (HIMEDIA) making a total of 50µl of the reaction mixture (Table 4). The DNA from the vaccine was used as a positive control and the rectal swab from a healthy dog was used as negative control.

Table 4: PCR Master Mix

Components	Volume
10X PCR buffer (with 15mM $MgCl_2$)	5.0µl
Forward Primer (20pm/µl)	1.0µl
Reverse Primer (20pm/µl)	1.0µl
dNTPs (10mM each)	1.0µl
Taq polymerase (5units/µl)	0.2µl
Template DNA	15.0µl
Nuclease free water	26.8µl
Total	50µl

After preparing the PCR mix, the reaction was put in a thermocycler (BioRad) which was subjected to the following thermocycling conditions i.e. for the step of denaturation a temperature of 94°C was kept for 60 seconds, for the step of annealing a temperature of 55°C was kept for 60 seconds and the extension step was set up at 72°C for 150 seconds and these were carried out for a total of 35 cycles (Table 5):

Table 5: Thermocycling conditions for PCR and Nested PCR

Steps	Temperature (°C)	Time	Cycles
Denaturation	94	60s	35
Annealing	55	60s	
Extension	72	150s	

After the completion of PCR, the PCR product was subjected to nested PCR by setting up a different PCR reaction mixture. To prepare the reaction mixture for nested PCR, 2.0µl of the PCR product obtained from the above procedure was added along with 2.5µl of 10X PCR buffer (with $MgCl_2$), 1.0µl each of forward primer (CPV 4) and reverse primer (CPV 6), 1.0µl of dNTP's, 0.2µl of Taq polymerase and 17.3µl of Nuclease free water making a total of 25µl of nested PCR reaction mixture (Table 6). Vaccine was used as the positive control and rectal swab from a healthy dog was made the negative control. Similar thermocycling conditions were setup for amplification (Table 5).

Table 6: Nested PCR Master Mix

Components	Quantity
10X PCR buffer (with $MgCl_2$)	2.5µl
Forward Primer (CPV-4)	1.0µl
Reverse Primer (CPV-6)	1.0µl
dNTP	1.0µl
Taq Polymerase	0.2µl
PCR Product	2.0µl
Nuclease Free Water (NFW)	17.3µl
TOTAL	25µl

Gel electrophoresis

The PCR product was run in a 1.5% gel in 10X TBE at 90 volts. The TBE was prepared by adding 10.8gm Tris hydroxyl methyl amino methane, 5.5gm boric acid (10X), 0.938gm EDTA and distilled water upto 100ml (Table 7).

Table 7: Preparation of TBE (Tris Borate EDTA)

Components	Quantity
Tris hydroxyl methyl amino methane	10.8gm
Boric acid	5.5gm
EDTA	0.938gm
Distilled Water	Upto 100ml

To make 1.5% TBE, 0.75gm agarose was dissolved in 50ml distilled water and heated in a microwave till the white crystals of agarose are completely dissolved to make a clear solution, 2.5ml ethidium bromide was added upon cooling. The comb was adjusted at one side of the gel tray while the mixture was poured into the gel tray and left for cooling. After the gel is prepared and is solid, the comb was removed and the gel was transferred into the tank filled with TBE. Load 2µl of the loading dye in a pipette and 10µl of the sample and fill the wells accordingly. The last well was filled with 3µl of ladder (Gene Ruler Ladder Plus 100bp, New England Biolabs, USA) and run at 90 volts till the gel was half run.

3.2.9 Ultracentrifugation/Purification of virus

After the production of virus in bulk to an approximate volume of 60ml each for CPV 2a (local isolate) and CPV 2b virus it was purified by ultracentrifugation. Ultracentrifugation of the cell-culture supernatant for both the isolates of CPV was done at National Research Center on Equines, Hisar (NRCE,HISAR).

- The 60 ml inoculate was transferred to the ultracentrifuge tubes which were put in the ultracentrifuge machine at 1,00,000g for 2hrs at 4°C.
- After ultracentrifugation was completed, the pellet was retained while the supernatant was discarded carefully
- 15 ml of PBS (pH 7.4) was added into the ultracentrifuge tube and the pellet was thoroughly dissolved and mixed in the PBS.
- Small aliquots of 1.0ml each of purified virus were made in eppendorf tubes.
- These aliquots were then stored at -20°C.

3.2.10 Protein estimation of purified virus

Protein estimation was done by A280nm method as it includes direct measurement and is the best method for pure proteins that contain Trp and Tyr residues. Therefore protein estimation for the purified virus CPV 2a and CPV 2b was done by using Nanodrop (Thermo Fisher, USA) at 280nm.

3.2.11 Raising of hyperimmune sera in rabbits

For raising hyper immune serum for the two CPV types (CPV 2a and CPV 2b) two rabbits were purchased from Paradise Rabbit Farm, Kurukshetra. The rabbits were aged between 8 months to one year, male and were a non-descript breed. The rabbits were kept at small animal house, GADVASU, Ludhiana.

The two rabbits were to be injected with purified virus of CPV 2a and CPV 2b type for raising hyper immune serum. 2.0 ml of virus suspension was injected I/M in the rabbits each for CPV 2a and CPC 2b. Then three injections were given I/V at weekly interval 1.0 ml each. One week after last injection blood was collected from rabbits and serum separated. The antibodies raised against CPV were confirmed by indirect ELISA. Finally then blood was collected from rabbits, serum separated and kept at -20°C for cross neutralization assay.

3.2.11 Confirmation of Hyper immune serum by Indirect ELISA

For the confirmation and to know the antibody titre in the hyperimmune serum raised in rabbits for the two CPV types viz. CPV 2a and CPV 2b, indirect ELISA was done using the INGEZIM PARVO CANINO 15.CPV.K1 Kit.

The procedure was followed as per the manufacturer's instructions with few modifications.

- **Preparation of serum sample:** The sera samples were diluted at 1/100 dilution in the diluents i.e. 5µl of serum was diluted in 0.5ml of diluent.
- **Preparation of washing buffer:** 10µl of washing solution was diluted in 90µl of distilled water and stored in refrigerator until used
- **Preparation of conjugate (anti-rabbit conjugate):** 1/100 dilution of conjugate was done by diluting 10µl of conjugate in 1ml of diluents.
- **Procedure**

i. 100µl of positive control serum available in the ELISA kit was added to the two wells of the first row of the plate.

ii. 100µl of the negative control serum (available in the kit) was added to the next two wells of the plate.

iii. Then 100µl of the diluted serum sample each of CPV-2a and CPV-2b was added in the wells in duplicate.

iv. The plate was sealed and incubated for 10 min at room temperature.

v. The plate was washed for 3 times using washing buffer.

vi. 100µl of conjugate (anti-rabbit conjugate 1:100) was added to each well

vii. The plate was sealed and incubated for 10 min at room temperature.

viii. The plate was washed 5 times with washing buffer.

ix. 100µl of substrate solution (available in kit) was added to each well and the plate was kept at room temperature for 5 min.

x. 100µl of stop solution (available in kit) was added to each well.

xi. The absorbances for each well were read with an ELISA reader (Thermo, USA) at 450nm within 5 min of addition of stop solution.

- **Calculation of antibody titre**

  Results Validation:

  The test is validated when:

  OD 450 Control (+) is higher than 1.2

  OD 450 Control (-) is lower than 0.15

Results Interpretation:

The ratio S/P (sample OD/control +OD) must be calculated. Samples with S/P lower than 0.15 should be considered negative and samples with S/P ratio higher than 0.15 should be considered positive to CPV antibodies.

The titre of the sample was calculated as:

$$Y \text{ (titre)} = 54\ (e^{4x})$$

Where, **e** is the base of natural logarithm (2.718282) and **x** the S/P ratio of the sample.

3.2.12 Estimation of TCID50 of the purified virus

The Tissue Culture Infective Dose 50 (TCID50) of both the purified virus i.e. CPV 2a and CPV 2b was calculated by **REED and MUENCH formula.**

To calculate the number of 50% infections or lethal doses per unit volume, the following procedure was carried out:

i. Add 900µl of PBS in the eppendorf tubes.

ii. To make 1:10 dilution of virus, 100µl of virus was mixed with 900µl PBS in the 1st tube

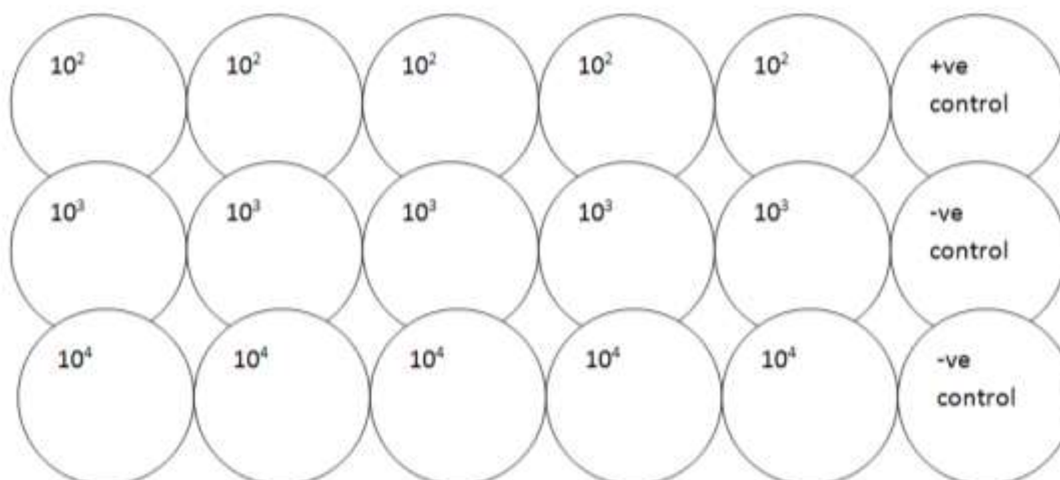
iii. Transfer 100µl from the 1st tube to 900µl PBS in the next tube and so on and discard 100µl from the last tube.

We have the following dilutions:

We have the following dilutions:	1:10	10^1
	1:100	10^2
	1:1000	10^3
	1:10000	10^4

For estimation of TCID50, MDCK cells were used. A 24-well cell culture plate with each well having complete monolayer of cells was used. The growth media was discarded from the wells of the cell culture plate and 1.0ml of each dilution of virus was added in the five wells of culture plate. Two 24 well plates were used one each for each type of CPV. The plates were then incubated for 1 and ½ hr at 37°C

with 5% CO2. After incubation the inoculums was discarded from the wells and 500µl of maintenance media (MM-DMEM) was added in each well. Two wells in the cell culture plate were kept as positive control in which undiluted virus was added and two wells as negative control in which only MM-DMEM was added. The plates were then incubated at 37°C with 5% CO2 further and observed daily for development of CPE. The results were read when CPE was observed in positive control wells.



Calculation of TCID50 by REED and MUENCH FORMULA

The CPE observed for each dilution (each dilution inoculated the five wells) was noted and TCID50 was calculated by using the formula:

$$\text{Proportional distance (E)} = \frac{\text{Mortality above 50\% - 50}}{\text{Mortality above 50\% - below 50\%}}$$

TCID50 = Log of dilution above 50% + (E * dilution factor)

3.2.13 Cross neutralisation test

For the cross neutralization test virus was kept constant and dilutions of serum were made. Therefore, 100 TCID50 of the virus was used for both the virus types i.e. CPV 2a and CPV 2b. The two fold serial dilution of the hyper immune serum produced against both the CPV types in rabbits were made and used in neutralization test. The following four sets of serum-virus combinations were used in the assay.

	SET I	SET II	SET III	SET IV
SERUM	2a	2a	2b	2b
VIRUS	2a	2b	2a	2b

For each set of serum-virus combination the following procedure was followed:

I. **SERUM PREPARATION**: The serum was prepared by first diluting it to 1:10 as it was very high in concentration. Therefore 100μl of serum was added to 900μl of PBS (pH 7.4) to make 1ml of diluents.

II. **SERUM DILUTION**: Two fold serial dilution of serum were prepared. The eppendorf tubes were first filled with 500μl of PBS and 500μl of serum was added from 1:10 dilution of serum in first tube. It was mixed and 500μl of diluted serum from first tube was transferred to the 2nd tube and so on till dilution the dilution of 1:16384 was achieved. Then 500μl was discarded from the last tube. So, finally the following dilutions of serum were obtained.

1:2	1:4	1:8	1:16	1:32
1:64	1:128	1:256	1:512	1:1024
1:2048	1:4096	1:8192	1:16384	DISCARD

III. **VIRUS DILUTION**: 1 TCID50 of each of the CPV type was achieved as in section 3.2.12. In cross neutralization test 100 TCID50 for both the CPV types was used. Therefore, 100 TCID 50 of the virus was prepared in PBS using the purified virus for both the virus types.

IV. **SERUM- VIRUS MIXTURE**: Serum- virus mixtures were prepared according to each set of serum and virus type. Equal volume (1.0ml) of each dilution of serum was mixed with 100 TCID50 (1.0ml) of the respective CPV type of the virus and incubated for 2 hours at 37°C.

V. **INOCULATION OF MDCK MONOLAYER**: A 24- well cell culture plate with monolayer of MDCK cells in wells of the plate was used for each set of serum-virus combination. The growth media (GM-DMEM) was discarded from the wells and each serum-virus dilution for each dilution of serum was inoculated 0.2 ml in wells of the cell culture plate i.e. five wells per dilution of serum. The plate was incubated at 37°C with 5% CO2 for one and a half hour. After incubation the serum-virus mixture was discarded and 500μl of maintenance media (MM-DMEM) was added in each well. The plates were incubated at 37°C with 5% CO_2 .

VI. **CONTROLS used in the experiment**: Three controls were used in the experiment:

Virus control: In this 100 TCID50 of the respective CPV type of virus was inoculated (0.2ml) in the well of the cell culture plate and after adsorption of the virus, it was replaced with 500µl of MM-DMEM.

Serum control: In this 1:10 dilution of the respective serum type was inoculated (0.2ml) in the well of the cell culture plate and after one and a half hour of incubation was replaced with 500µl of MM-DMEM.

Negative control: In this 500µl of MM-DMEM was added.

VII. **Observation of cytopathic effects**: The plates were observed daily for the development of cytopathic effects. The results were noted when CPE was observed in the virus control.

VIII. The reciprocal of the maximum dilution of serum which did not exhibit CPE was the neutralization titre of the serum for the respective CPV type of the virus.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Real Time PCR typing for identifying CPV subtypes

A total of 178 samples available in the Department of Veterinary Microbiology, COVS, GADAVSU, Ludhiana collected from the various regions of northern India viz. Punjab (n=119), Assam (n=36), Delhi (n=11), Jammu (n=6) and Chandigarh (n=6) were subjected to Real Time PCR typing for detection of three CPV antigenic types (CPV 2, CPV 2a and CPV 2b) (Table 8). Out of a total of 178 samples; 133 samples were found positive for Canine Parvovirus with a percent positivity of 74.71%. Out of 133 positive samples; a total of 27 cases were positive for CPV 2, 86 were positive for CPV 2a and 20 were positive for CPV 2b (Table 9 and Fig. 1). Therefore CPV 2a antigenic type of Canine parvovirus is most prevalent followed by CPV 2 and CPV 2b.

However, when the results were thoroughly observed, some cases were found to be positive for more than a single subtype simultaneously. Therefore the number of dogs positive for CPV 2 alone were 3, for CPV 2a there were 61 cases, for CPV 2b there were 7 cases, number of positive cases for both CPV 2 and 2a were 12, for 2a and 2b there was just 1 case, no case was positive for CPV 2 and 2b simultaneously while 12 cases were positive for CPV 2, 2a and 2b altogether (Table 10 and Fig. 2).

Table 8: Real Time PCR results for CPV 2, 2a and 2b

Samples	CPV 2	CPV 2a	CPV 2b
ASSAM			
A1	-	-	-
A2	-	Positive	-
A3	-	-	-
A4	-	Positive	-
A5	-	Positive	-
A6	-	-	-
A7	-	-	-
A8	-	-	-

Samples	CPV 2	CPV 2a	CPV 2b
A9	-	-	-
A10	-	-	-
A11	-	-	-
A12	-	-	-
A13	-	-	-
A14	-	Positive	Positive
A15	-	-	-
A16	-	-	-
A17	-	-	Positive
A18	-	-	Positive
A19	-	-	-
A20	-	-	Positive
A21	-	-	-
A22	-	-	-
A23	-	-	-
A24	-	-	-
A25	-	-	-
A26	-	Positive	-
A27	-	Posiitve	-
A28	-	-	-
A29	-	-	-
A30	-	Positive	-
A31	-	Positive	-
A32	-	-	-
A33	-	-	-
A34	-	Positive	-
A35	-	-	-
A36	-	Positive	-
PUNJAB			

Samples	CPV 2	CPV 2a	CPV 2b
G1	-	-	-
G2	-	Positive	-
G3	-	-	-
G4	-	Positive	-
G5	-	-	-
G6	-	-	-
G7	-	-	Positive
G8	-	-	Positive
G9	-	Positive	-
G10	-	-	-
G11	-	Positive	-
G12	-	Positive	-
G13	-	-	-
G14	-	-	-
G15	-	-	-
G16	-	-	-
G17	-	-	-
G18	-	Positive	-
G19	-	Positive	-
G20	-	-	-
G21	-	-	-
G22	-	Positive	-
G23	-	-	-
G24	-	-	-
G25	-	-	-
G26	-	-	Positive
G27	-	-	-
G28	-	Positive	-
G29	-	-	-

Samples	CPV 2	CPV 2a	CPV 2b
G30	-	Positive	-
G31	-	Positive	-
G32	-	Positive	-
G33	-	-	-
G34	-	Positive	-
G35	-	-	-
G36	-	-	-
G37	-	-	-
G38	-	-	-
G39	-	-	-
G40	Positive	-	-
G41	-	-	-
G42	Positive	-	-
G43	-	Positive	-
G44	Positive	Positive	-
G45	-	-	-
G46	-	-	-
G47	Positive	-	-
G48	-	-	-
G49	-	-	-
G50	-	-	-
G51	Positive	Positive	-
G52	Posiitve	Positive	-
G53	Positive	Positive	Positive
G54	Posiitve	Positive	Positive
G55	-	Positive	-
G56	-	Positive	-
G57	-	Positive	-
G58	-	-	-

Samples	CPV 2	CPV 2a	CPV 2b
G59	-	-	-
G60	Positive	Positive	-
G61	-	-	-
G62	Positive	Positive	-
G63	-	-	-
G64	-	Positive	-
P1	-	Positive	-
P2	-	-	-
P3	-	-	-
P4	-	Positive	-
P5	-	Positive	-
P6	-	Positive	-
P7	-	Positive	-
P8	-	Positive	-
P9	-	-	-
P10	-	-	-
P11	-	-	-
P12	-	-	-
P13	-	-	-
P14	-	Positive	-
P15	-	Positive	-
P16	-	-	-
P17	-	-	-
P18	-	-	-
P19	-	Positive	-
P20	-	-	-
P21	-	-	-
P22	-	Positive	-
P23	-	-	-

Samples	CPV 2	CPV 2a	CPV 2b
P24	-	Positive	-
P25	-	-	-
P26	-	-	-
P27	Positive	Positive	-
P28	-	Positive	-
P29	Positive	Positive	Positive
P30	Positive	Positive	-
P31	-	-	-
P32	-	Positive	-
P33	-	Positive	-
P34	Positive	Positive	Positive
P35	-	-	-
P36	-	-	-
P37	-	Positive	-
P38	-	Positive	-
P39	-	Positive	-
P40	-	-	-
P41	-	Positive	-
P42	-	-	-
P43	-	Positive	-
P44	-	-	-
P45	-	Positive	-
P46	Positive	Positive	Positive
P47	-	Positive	-
P48	Positive	Positive	-
P49	-	Positive	-
P50	-	Positive	-
P51	-	Positive	-
P52	-	Positive	-
P53	Positive	Positive	Positive

Samples	CPV 2	CPV 2a	CPV 2b
P54	**-**	**Positive**	**-**
P55	**-**	**Positive**	**-**
DELHI			
D1	**-**	**Positive**	**-**
D2	**Positive**	**Positive**	**Positive**
D3	**Positive**	**Positive**	**-**
D4	**Positive**	**Positive**	**Positive**
D5	**-**	**-**	**-**
D6	**Positive**	**Positive**	**Positive**
D7	**Positive**	**Positive**	**Positive**
D8	**Positive**	**Positive**	**Positive**
D9	**-**	**-**	**-**
D10	**-**	**Positive**	**-**
D11	**-**	**Positive**	**-**
JAMMU			
J1	**Positive**	**Positive**	**-**
J2	**-**	**Positive**	**-**
J3	**Positive**	**Positive**	**Positive**
J4	**-**	**-**	**Positive**
J5	**Positive**	**Positive**	**-**
J6	**-**	**Positive**	**-**
CHANDIGARH			
C1	**-**	**Positive**	**-**
C2	**-**	**-**	**-**
C3	**-**	**Positive**	**-**
C4	**-**	**-**	**-**
C5	**-**	**-**	**-**
C6	**Positive**	**Positive**	**-**
TOTAL	**27**	**86**	**20**

Table 9: Number of Canine Parvovirus positive samples

Total number of cases	**178**
Total CPV 2 positive	**27**
Total CPV 2a positive	**86**
Total CPV 2b positive	**20**
Total cases positive	**133**

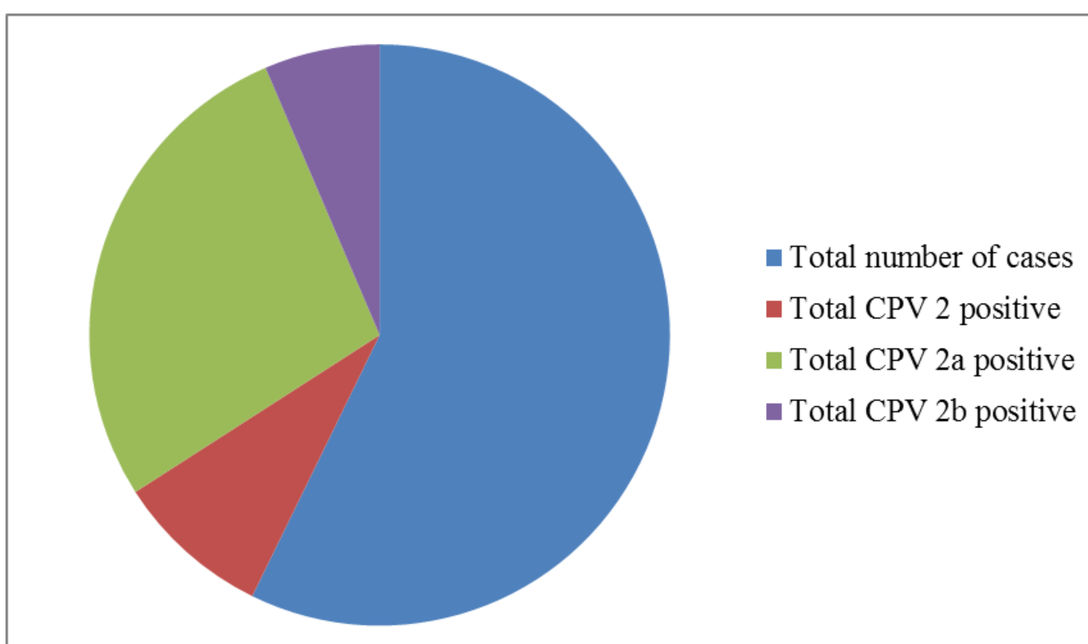


Fig. 1: Pie Chart representation of Real Time PCR results

Table 10: Number of cases positive for CPV subtypes simultaneously

CPV 2 only	**3**
CPV 2a only	**61**
CPV 2b only	**7**
CPV 2 & 2a	**12**
CPV 2 & 2b	**0**
CPV 2a & 2b	**1**
CPV 2, 2a & 2b	**12**

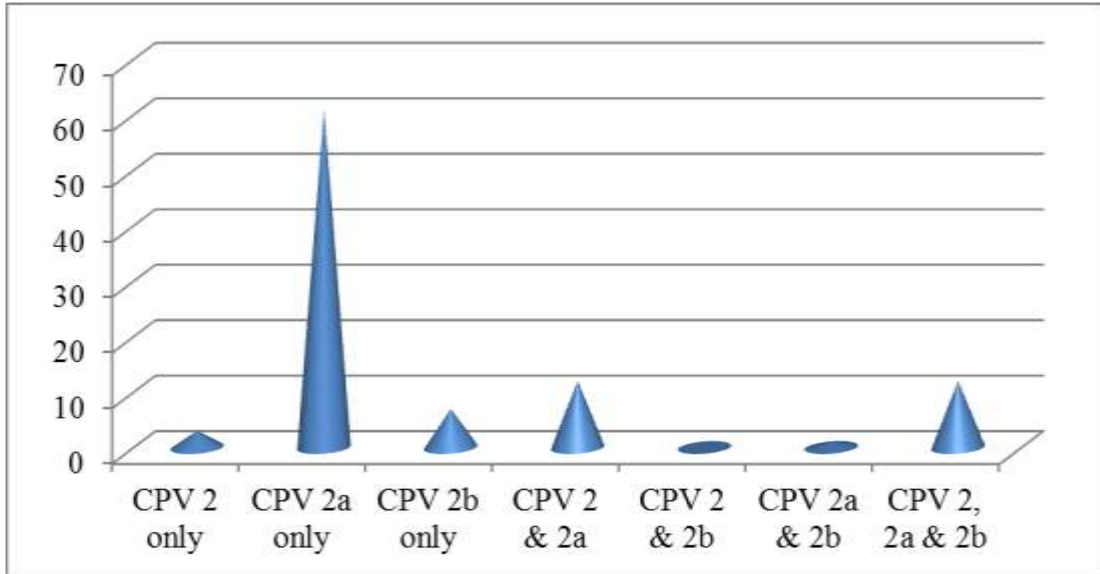


Fig. 2: Graphical representation of number of cases positive for CPV subtypes simultaneously

When the distribution of three CPV types (CPV 2, CPV 2a and CPV 2b) in various regions under study was analysed; it was observed that in Assam ten samples were positive for CPV 2a type and four were positive for CPV 2b; in Punjab 17 samples were positive for CPV 2, 59 samples were positive for CPV 2a and nine samples were positive for CPV 2b. Similarly in Delhi six samples were positive for CPV 2, nine samples were positive for CPV 2a and five samples were positive for CPV 2b, one sample collected from Chandigarh was positive for CPV 2 type and three samples positive for CPV 2a and finally the three samples collected from Jammu were positive for CPV 2, five samples were positive for CPV 2a and two samples were found positive for CPV 2b (Table 11 and Fig. 3). Therefore, the region wise distribution of CPV types indicated that CPV 2a type of Canine Parvovirus was most prevalent followed by CPV 2 and CPV 2b type.

Table 11: Region wise distribution of CPV subtypes

State	CPV 2	CPV 2a	CPV 2b
Assam	0	10	4
Punjab	17	59	9
Delhi	6	9	5
Chandigarh	1	3	0
Jammu	3	5	2

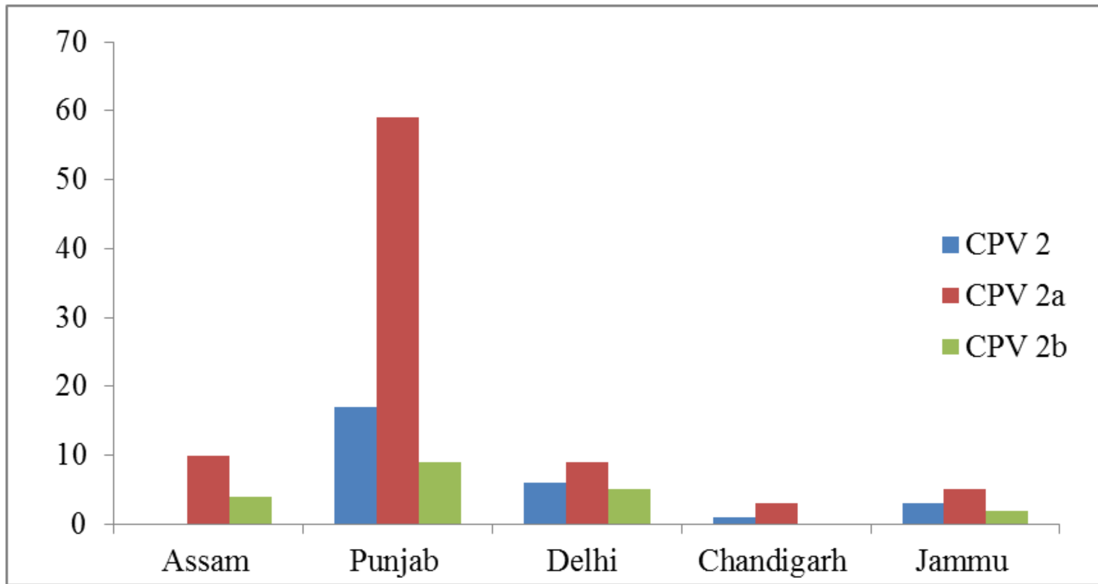


Fig. 3: Graphical representation of distribution of CPV subtypes region wise

4.1.2 Determination of end point of RT-PCR

The final result of RT-PCR was detected by the graph displayed at the end of procedure in CFX Manager Version 3.1. Depending on the fluorescence emitted of a particular wavelength by the respective fluorophore the curve of the graph is determined also giving us the RFU value, cut off value and therefore the result can be calculated. The graph of the positive samples for CPV 2, 2a and 2b is given in Figs. 4, 5 and 6, respectively.

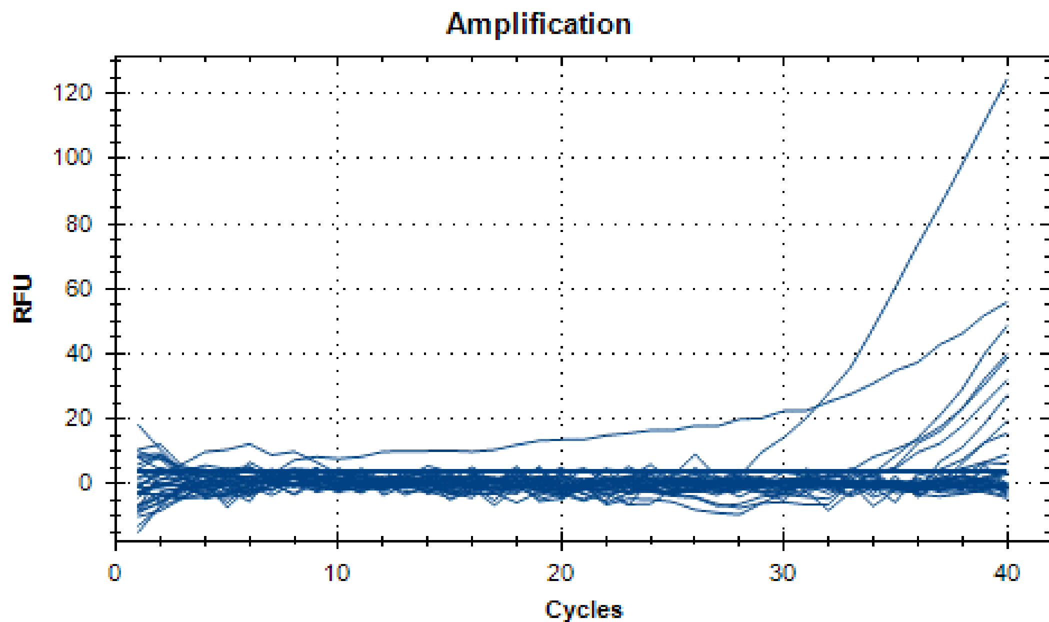


Fig 4: Real-time PCR graph for positive sample for CPV 2

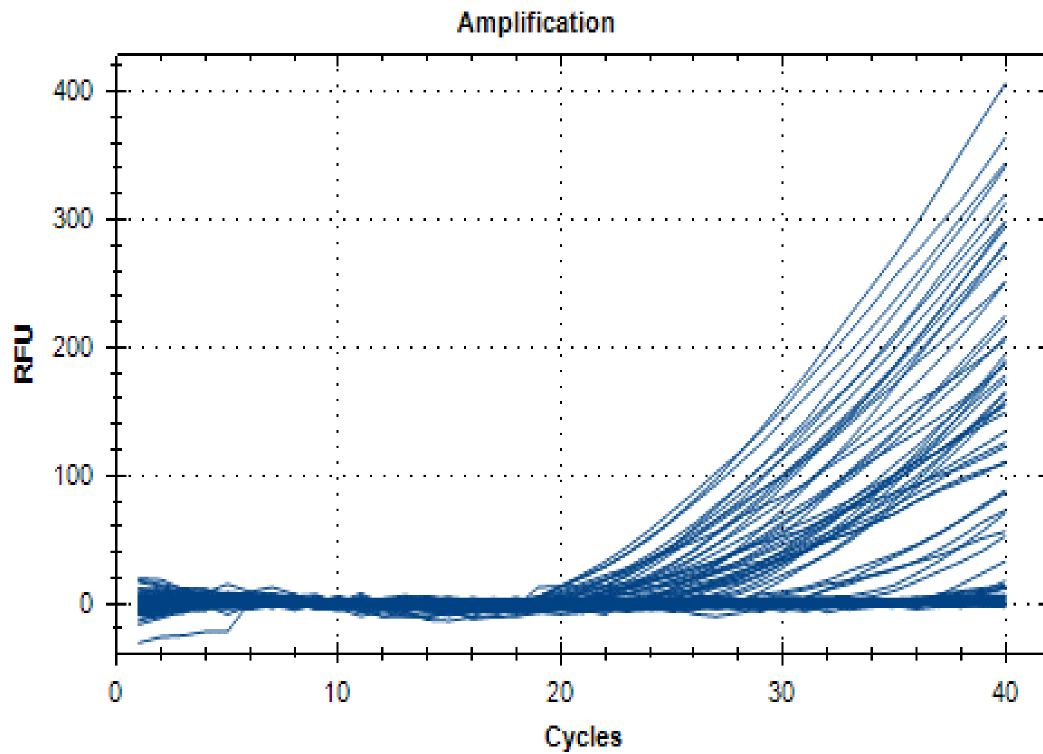


Fig 5: Real-time PCR graph for positive sample for CPV 2a

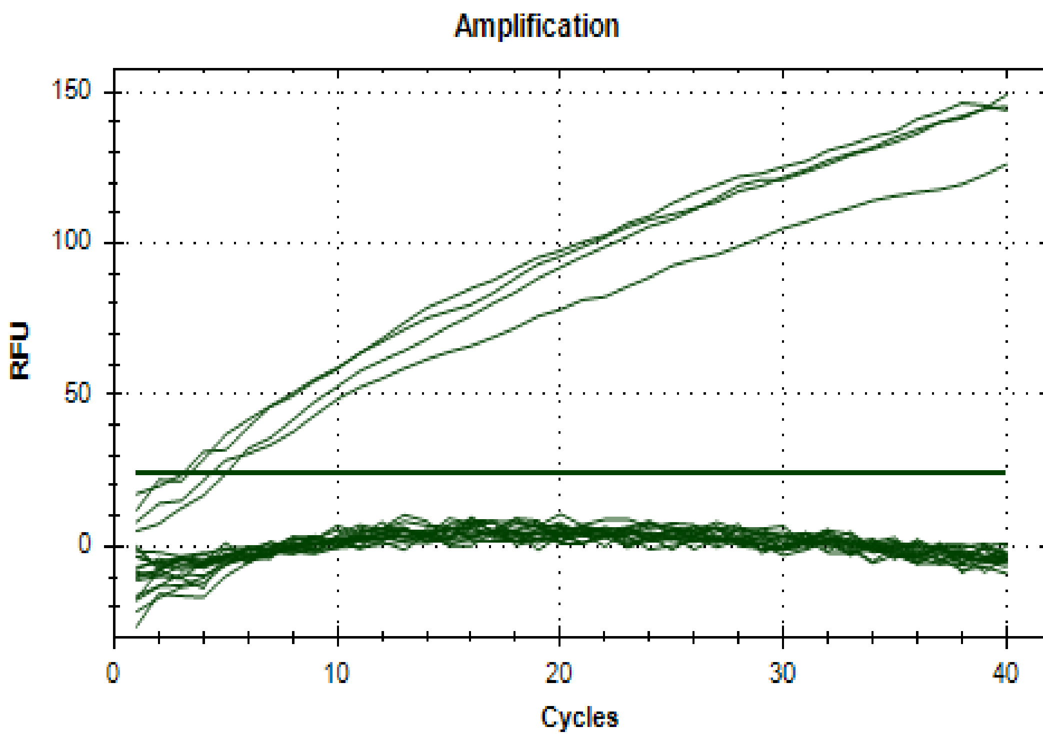


Fig 6: Real-time PCR graph for positive sample for CPV 2b

Scientists throughout the world have used real time PCR for the studies on Canine Parvovirus.

Using real-time PCR, prolonged fecal shedding of the Glu-426 mutant DNA was observed by Decaro *et al* in 2005. He noticed that in previous studies, viral shedding in the feces had been detected up to 12 days by virus isolation in cell culture (Pollock RV, 1982) and for up to 7 days by HA (Carmichael *et al* 1980). It was studied that the higher sensitivity of real-time PCR allowed the detection of viral nucleic acid for a much longer period, even when titers dropped to about 104 DNA copies/mg of feces toward the end. Further in 2013, Streck *et al* discovered that the real-time PCR represents an upgraded and useful tool to identify and quantify canine and feline parvoviruses in different sample matrices like faeces and sera.

Kumar and Nandi in 2010 took 47 faecal samples from dogs suspected of CPV 2 infection were analyzed by real-time PCR, haemagglutination (HA) assay and by a conventional PCR out of which 24, 20 and 22 samples were found positive for CPV 2, respectively. When the results of three different assays were compared, they revealed that real-time PCR is more sensitive than HA and conventional PCR and real-time PCR also allowed the detection of low titers of CPV 2 in infected dogs. Decaro *et al* in 2008 characterized CPV-2c strain by means of real-time PCR assays using minor groove binder probes. Touihri *et al* in 2009 carried out a study in Tunisia and the investigation showed that most of clinically presumed CPV infections were confirmed by classical or real-time PCR.

Twelve dogs were found dead as consequence of natural infection caused by canine parvovirus (CPV) and the details of the respective subtypes was that 4 animals were dead because of each type 2a, 2b and 2c. Decaro *et al* in 2007 also investigated for determining the viral DNA loads in different tissue samples by means of a real-time PCR assay concluding that the highest titres were observed in the lymphoid tissue and the lowest loads in the urinary tract.

The parvovirus and coronavirus strains detected in the feline and canine samples were characterised by Decaro *et al* in 2010 by genotype-specific real-time PCR or RT-PCR assays. Prediction of the parvovirus type was obtained by a panel of real-time PCR assays using minor groove binder (MGB) probes which were able to discriminate between: (i) FPLV and CPV (Decaro *et al* 2008b) (ii) CPV-2a and 2b or

CPV-2b and 2c (Decaro *et al* 2006b) and (iii) CPV vaccine and field strains (Decaro *et al* 2006a, Decaro *et al* 2006d).

Kaur *et al* (2016) carried out a study for identification as well as antigenic typing of CPV using a *de novo* multiplex real time PCR. From the study it could be concluded that the developed multiplex real time PCR assay could be used for rapid detection of CPV as well as typing of its three antigenic types.

4.2 To identify antigenic relationship of canine parvovirus types using *in-vitro* cross-neutralisation assay

4.2.1 Production of virus in bulk in MDCK cell Line

The antigenic relationship of two CPV types; CPV 2a and CPV 2b was studied using cross-neutralisation assay. For this the isolates of both types were produced in bulk in MDCK cell line. The CPV 2a isolate was available in the Department of Veterinary Microbiology, COVS, GADVASU, Ludhiana and the CPV 2b isolate was procured from Department of Veterinary Biotechnology, College of Veterinary Science, PVNR TVU, Hyderabad. For production of virus in bulk MDCK cell line was cultured and maintained in DMEM growth (10% FBS) and maintenance (2% FBS) media. The complete monolayer of MDCK cells was obtained within 48 to 72 hours. On Day 1, the cells appeared shining, globular in shape until monolayer was formed (Fig. 7). On day 2, adherence began and the adhered cells were epitheloid in shape (Fig. 8). On day 3, monolayer was complete (Fig. 9).

4.2.1.1 Virus inoculation in MDCK cells

For production of virus in bulk both the isolates of the virus were inoculated into the MDCK cells and the cytopathic effects were observed. On day 1, there were some slight changes observed in the cells (Fig. 10). On the second day of virus inoculation, peculiar rounding was observed in the cells which indicated that the cells were infected from canine parvovirus (Fig. 11). Finally, on day 3, the rounding disappeared and the cells were detached and the appearance of the cells started to become distorted (Fig. 12). After the cytopathic effects were observed the virus was harvested from the MDCK cells by three cycles of alternate freezing and thawing. The cell culture supernatant thus obtained was further passaged in MDCK cells to obtain virus for both the isolates of CPV in bulk. Therefore approximately 60 ml of virus was produced for both CPV 2a and CPV 2b types.

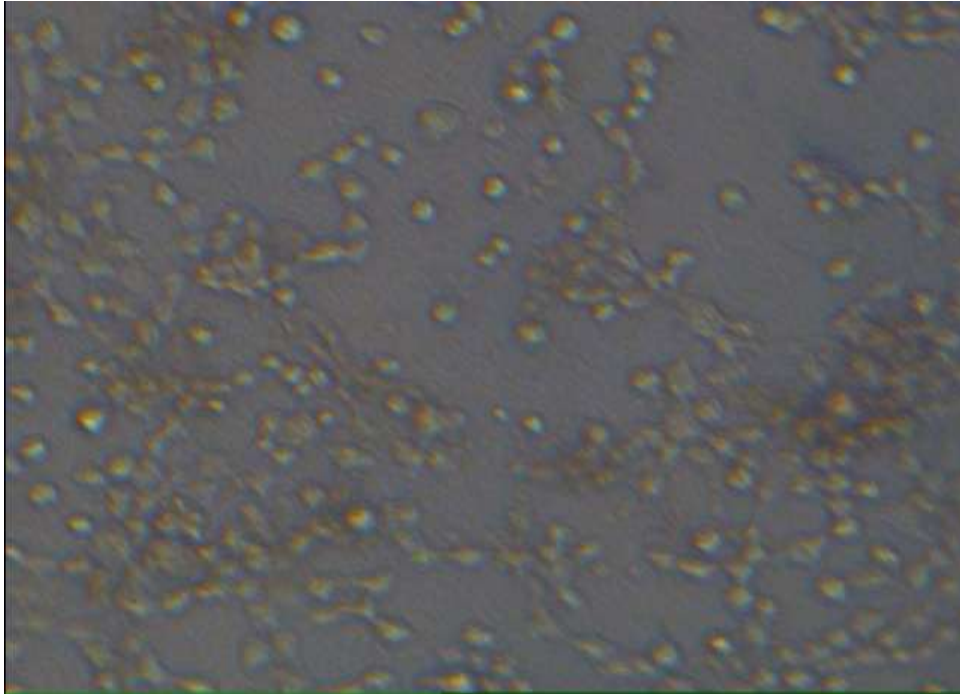


Fig 7: Appearance of MDCK cells on day1 (20x magnification)

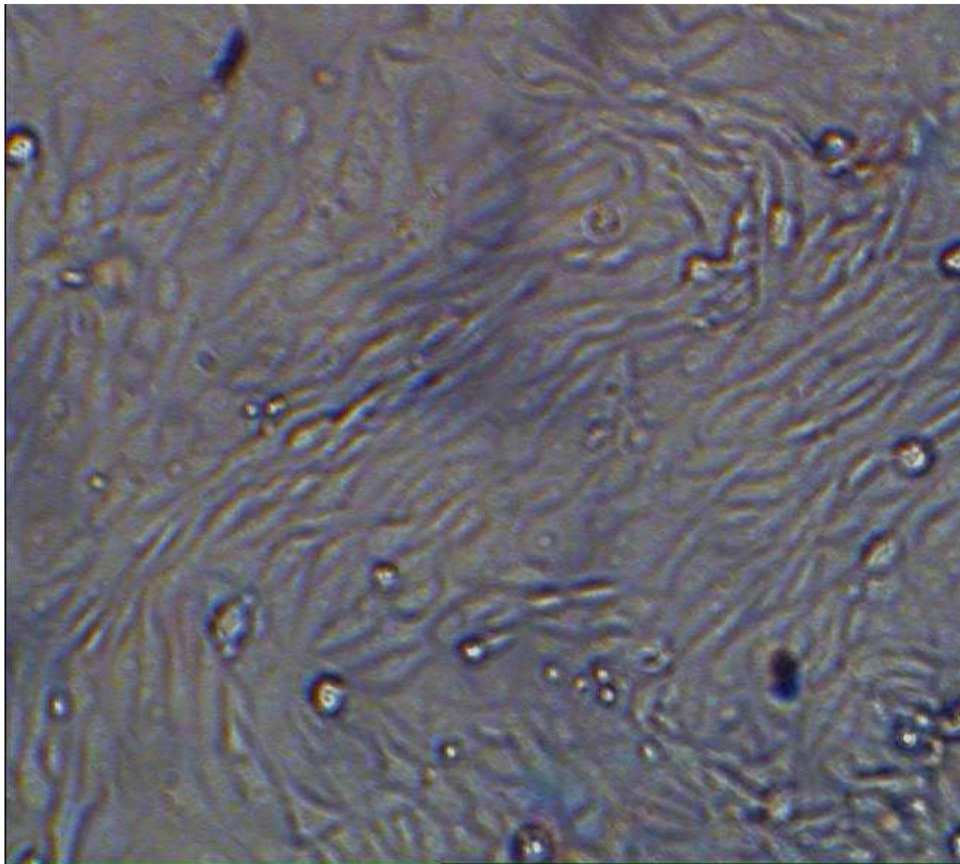


Fig 8: Appearance of MDCK cells on day 2 (20x magnification)

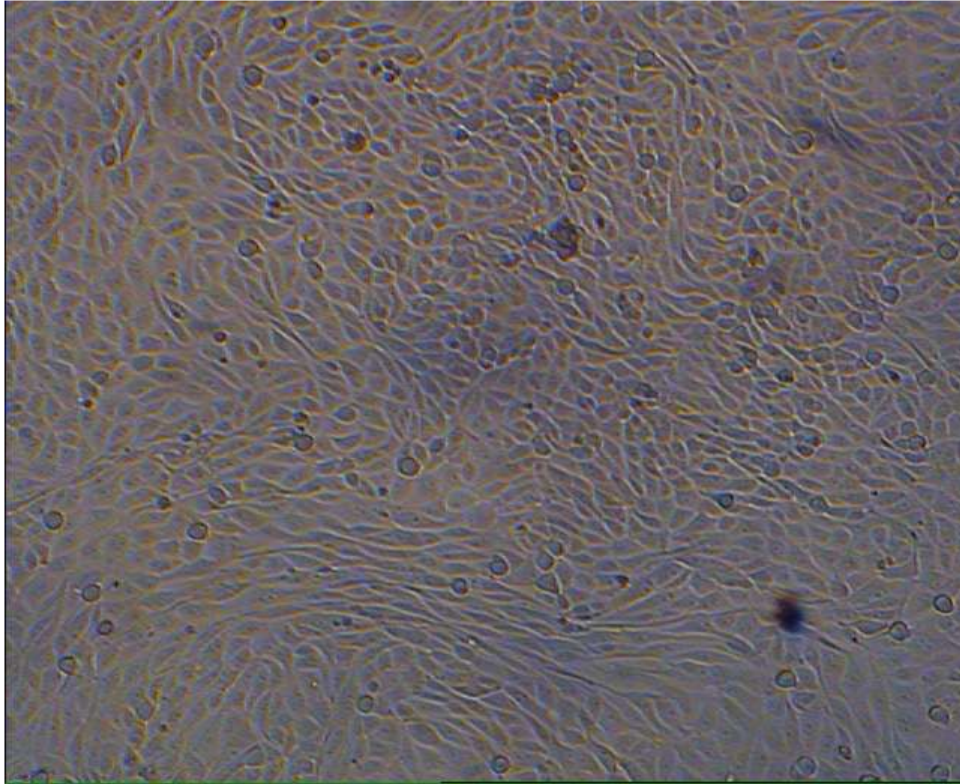


Fig 9: Appearance of MDCK cells on day 3 (10x magnification)

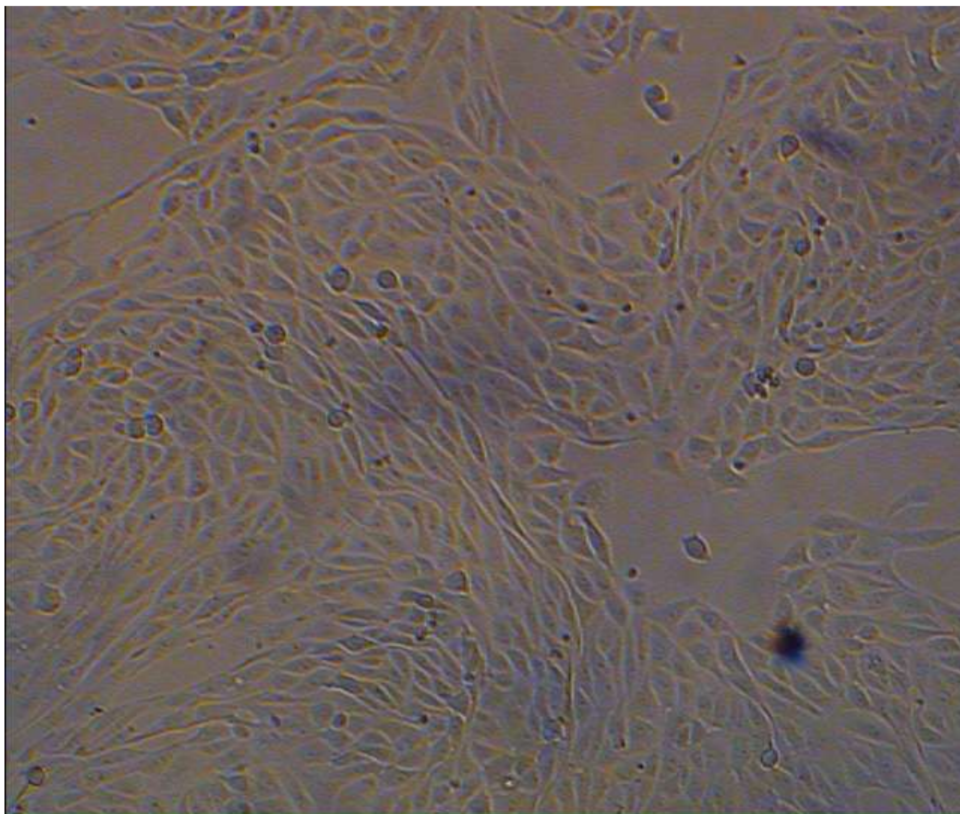


Fig 10: Day1 of inoculation of virus (10x magnification)

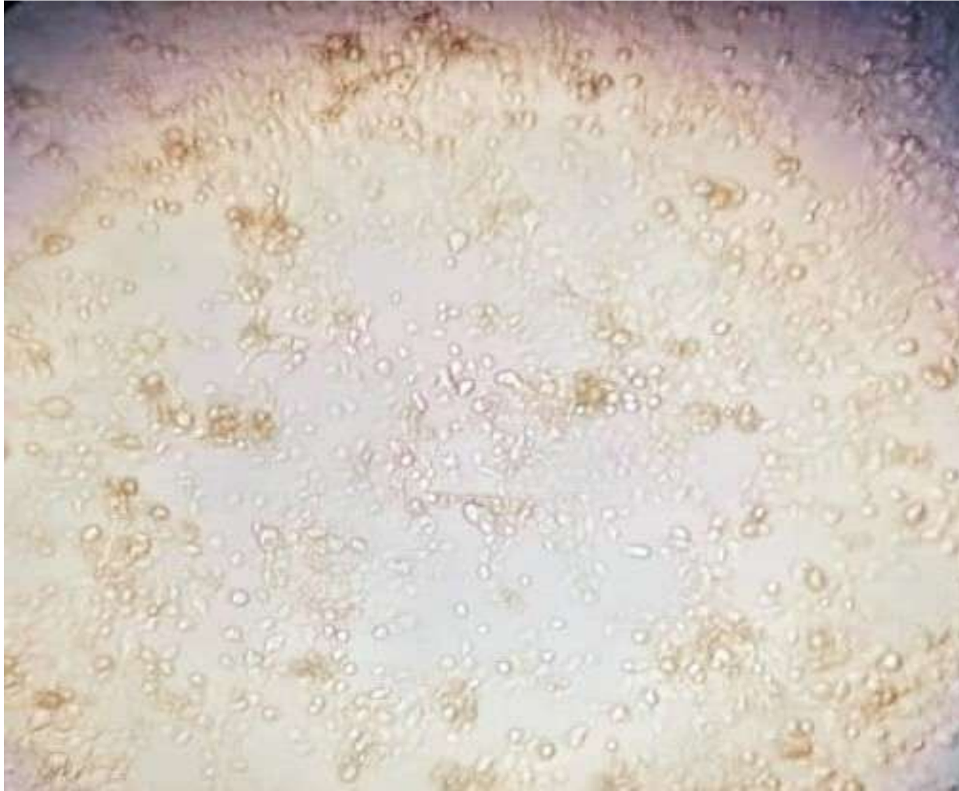


Fig 11: Cytopathic effect rounding is observed in cells (10x magnification)

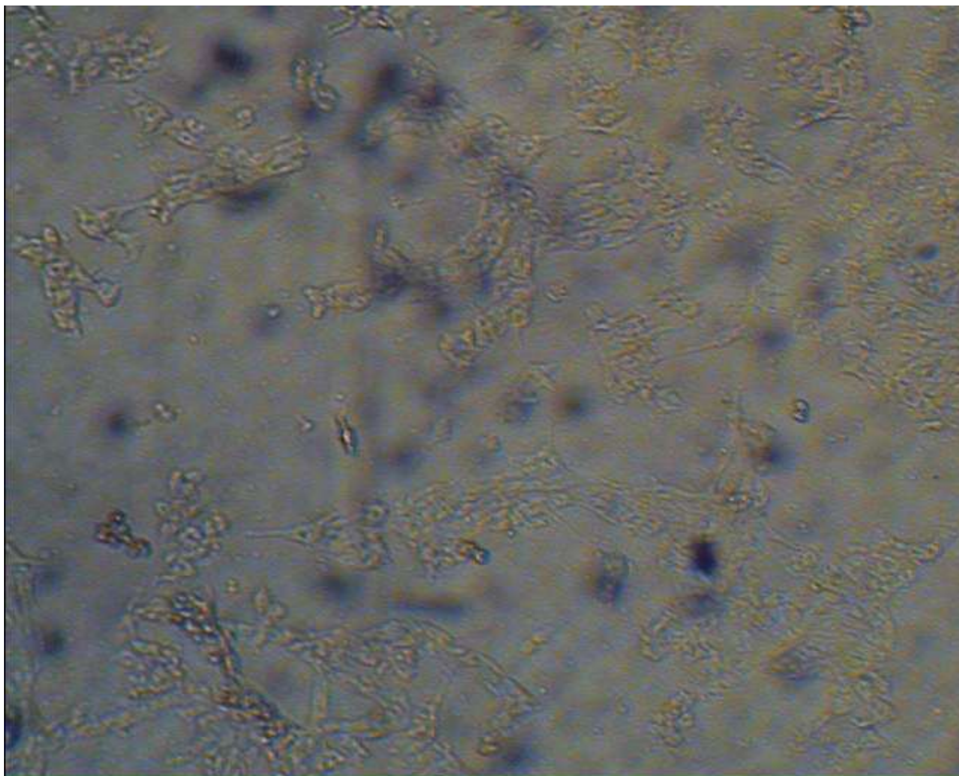


Fig 12: Detachment observed in cells on day 3 post inoculation (10x magnification)

Mochizuki *et al* in 2002 recovered viruses from three PCR-positive rectal specimens by using WRCC/3873D and MDCK cells.

MDCK cells were grown in 25cm cell culture plastic flask containing Dulbecco's MEM medium with 10 % fetal calf serum by Kumar *et al* in 2010 and were used to isolate the virus by adsorption method.

Three faecal samples which were found positive in PCR were subjected to blind passages in MDCK cells by Kumar *et al* in 2010. In the first 3 passages, no cytopathic effects (CPE) were seen. However, from 4th passage onwards, MDCK cells exhibited CPE which is characterized by rounding of cells, granulation and aggregation of cells after subsequently increasing to 72 hpi and widely distributed in whole monolayer. Similar cytopathic effects were observed in the present study in MDCK cells inoculated with the virus.

For isolation of CPV in living cells, Sharma *et al* in 2016 grew three representative virus isolates of antigenic variants in Madin-Darby canine kidney (MDCK) cell line. It was grown to confluence in Minimal Essential Medium (MEM) containing 10% fetal calf serum at 37°C with 5% CO_2. The growth medium was decanted, and 0.1 ml of viral inoculum was added to a 25 cm^2 tissue culture flask after the monolayer was 80-90% confluent.

MDCK cells were grown in 25 cm2 cell culture plastic flask containing Dulbecco's minimum essential medium (DMEM) with 10 % fetal calf serum (FCS) and used to isolate the virus by adsorption method by Kumar *et al* in 2010. When cell monolayer was confluent to 70% in cell culture flask, it was infected with 0.5 ml of processed faecal sample as inoculums. The presence of CPV in the cell culture was determined by presence of cytopathic effect (CPE) or by detecting haemagglutinin titer by HA assay. Therefore, many researchers have used MDCK cells for growing Canine Parvovirus as was used in this study.

4.2.1.2 Confirmation of cell culture supernatant for Canine Parvovirus

The cell culture supernatant obtained after freezing and thawing cycles was confirmed for CPV by PCR and nested PCR. The DNA was extracted from the cell culture supernatant of each of the isolate and subjected to PCR. The band for both the isolates i.e. CPV 2a and CPV 2b was observed at 1198 bp (Fig. 13). Further when the PCR product of both isolates of CPV was subjected to nested PCR, the band was

observed at 548bp length (Fig. 14). Thus confirming the presence of Canine Parvovirus in cell culture supernatant.

A polymerase chain reaction (PCR) for the detection of canine parvovirus (CPV) was developed by Hirasawa *et al* in 1994. The nested PCR with a double-nested primer pair (inner primer pair) was designed to increase the sensitivity and specificity of the reaction. The sequences of the PCR primer pairs were selected from the conserved region in the CPV VP1/VP2 gene.

A nested polymerase chain reaction (n-PCR) was developed by Kumar *et al* in 2011 using published pCPV-2ab as external primer set and self designed and custom synthesized pCPV-2N as internal primer set for the detection in faecal samples of dogs. Out of a total of 52 faecal samples, 27 and 31 were tested positive by one step PCR and n-PCR, respectively. Ten-fold dilution of known cell culture supernatants was also tested and it was concluded that one step PCR had a detection limit of 10^{-5} dilution, whereas nested-PCR had up to 10^{-8} dilution. Thus, the nested-PCR seemed to be a more sensitive, specific and practical method for the detection of CPV in faecal samples.

The aim of the study conducted by Kaur *et al* in 2015 was to isolate *Canine parvovirus* (CPV) from suspected dogs on madin darby canine kidney (MDCK) cell line and its confirmation by polymerase chain reaction (PCR) and nested PCR (NPCR). A 1710bp product was amplified when the samples and the two commercially available vaccines were subjected to PCR for amplification of VP2 gene. The DNA extracted from the samples was subjected to PCR and NPCR confirmed CPV by amplifying a 1198 bp product in PCR and 548 bp product in NPCR.

In the study conducted by Costa *et al* in 2005, the genomic types of canine parvovirus (CPV) circulating in the State of Rio de Janeiro, Brazil, from 1995 to 2001, were investigated using the polymerase chain reaction assay (PCR). A total of 78 faecal samples were confirmed positive for canine parvovirus. To distinguish the old (CPV-2) and new types of virus (CPv-2a or CPV-2b), PCR was carried out with differential pairs of primers.

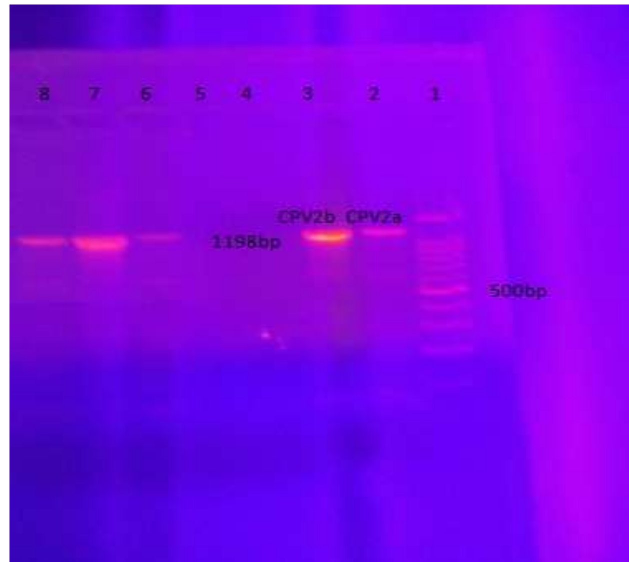


Fig. 13: PCR for confirmation of CPV in cell culture supernatant.

Lane 1: 500bp plus DNA ladder, Lane 2: CPV 2a isolate, Lane 3: CPV 2b isolate, Lane 4: Negative control, Lane 5, 6, 7, 8: other CPV samples.

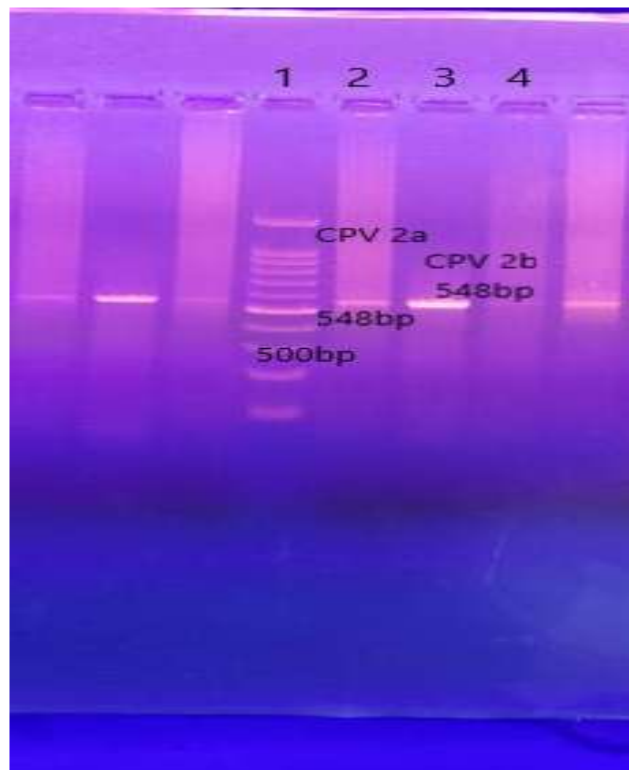


Fig. 14: Nested PCR for confirmation of CPV in cell culture

Lane 1: DNA ladder, lane 2: CPV 2a isolate, lane 3: CPV 2b isolate, lane 4: negative control

4.2.2 Protein estimation of the purified virus

After the production of virus in bulk to approximately 60 ml for each of the CPV 2a and CPV 2b types, the purification of the virus was done by ultracentrifugation at 1,00,000g for 2 hours. The pellet was obtained (Fig. 15) which was suspended in PBS. The protein estimation of the purified virus was done by using Nanodrop at 280nm.The protein concentration for the purified virus CPV 2a was found to be 93µg/ml and for the purified virus CPV 2b was found to be 531µg/ml.

4.2.3 Raising of hyper immune serum and confirmation by Indirect ELISA

The purified virus of both the CPV types (CPV 2a and 2b) was used to raise hyperimmune serum in rabbits. After following the whole protocol, blood was collected from rabbits by intra cardiac route (Fig. 16). The serum was separated from blood and subjected to Indirect ELISA for confirmation and to estimate antibody titre (Fig. 17 and Table 12).

Antibody titre for CPV 2a= 99606.24

Antibody titre for CPV 2b= 86940.955

Calculations:

Table 12: Results of Indirect ELISA for CPV 2a and CPV 2b

	OD Values	Average
CPV 2a	3.426	3.452
	3.478	
CPV 2b	3.314	3.377
	3.440	
Positive control	2.067	1.829
	1.591	
Negative control	0.040	0.037
	0.034	

Our results are validated as OD 450 for Positive Control is higher than 1.2

OD 450 for Negative Control is lower than 0.15

Calculating titre for CPV 2a:

Ratio S/P = OD of Sample/ OD of Postive Control = 3.452/ 1.829 = 1.88

$$Y \text{ (titre)} = 54\ (e^{4x}) = 54\ (2.718282^{4*1.88})$$

where e= 2.718282 and x= S/P ratio of sample

$= 54\ (2.718282^{7.52})$

$= 54 \times 1844.56$

$=$ **99606.24**

Calculating titre for CPV 2b:

Ratio S/P = 3.377/1.829 = 1.84

Y (titre) $=54(e^{4x}) = 54(2.718282^{4*1.84})$

$= 54\ (2.718282^{7.36})$'

$= 54 \times 1610.0177$

$=$ **86940.95**

Therefore, the titre for CPV 2a was calculated as 99606.24 and for CPV 2b 86940.95

4.2.4 Estimation of $TCID_{50}$ of the purified virus (CPV 2a and CPV 2b)

The $TCID_{50}$ of the purified virus was calculated by Reed and Muench formula (Table 13 and 14) in the 24 well cell culture plate (Fig. 18). For this ten fold serial dilution of the virus was done and four dilutions were used 10^1 to 10^4. Each dilution of the virus was inoculated in 5 wells of MDCK cells to observe the CPE produced.

Calculation of TCID 50 using Reed and Muench formula

Table 13: $TCID_{50}$ for CPV 2a

Dilutions	CPE positive	CPE negative	Cumulative Frequency		Ratio	Percentage
10^1	5	0	17	0	17/17	100%
10^2	5	0	12	0	12/12	100%
10^3	4	1	7	1	7/8	87.5%
10^4	3	2	3	3	3/6	50%

Here 50% is at 10^4. Therefore 1 $TCID_{50} = 10^4/0.1$ml

Titre $= 10^4 * 10^1 = 10^5$/ml

100 $TCID_{50} = 10^2 / 10^5 = 10^3$/ml

Table 14: $TCID_{50}$ for CPV 2b

Dilutions	CPE positive	CPE negative	Cumulative Frequency		Ratio	Percentage
10^1	5	0	15	0	15/15	100%
10^2	4	1	10	1	10/11	90.9%
10^3	4	1	6	2	6/8	75%
10^4	2	3	2	5	2/7	28.5%

Fig. 15: Virus pellet observed after ultracentrifugation

Fig. 16: Collection of blood from rabbits

Fig. 17: Confirmation of antibody titre of hyperimmune serum by Indirect ELISA.

Fig 18: The 24 well cell culture plate depicting $TCID_{50}$.

According to REED and MUENCH FORMULA:

$$\text{Proportional distance (E)} = \frac{\text{mortality above 50\% - 50}}{\text{Mortality above 50\% - below 50\%}} = \frac{75-50}{75-28.5} = 0.53$$

= Log of dilution above 50% + (e * dilution factor)

= 3 + (0.53*1) = 3 + 0.53 = 3.53

= 1 $TCID_{50}$ = log $10^{3.53}$/ 0.1ml

= 1 $TCID_{50}$ = log $10^{4.53}$/ ml

= **100 $TCID_{50}$ = log $10^{2.53}$/ml**

In a study conducted by Kumar *et al* in 2010 the optimum dilutions of capture and tracing antibodies were selected by check-board titration against fixed dilution of CPV antigens with different dilution of rabbit and guinea pig hyperimmune serum and results so obtained were at a 1:1 600 dilution for the capture antibody and a 1:400 dilution for the tracing antibody with 1:2 dilution of the positive antigen and the negative antigen.

To study the subcellular localization and tissue tropism of Canine Parvovirus He *et al* in 2012 titrated the CPV suspensions using 50% tissue culture infective dose (TCID50) assay (LaBarre and Lowy, 2001). Cells were seeded on 24 well plate, incubated for 1 h at 37°C and inoculated with CPV ($10^{3.65}$ TCID50 per well) and were incubated for 12 h after inoculation.

To investigate the pathogenicity of Canine Parvovirus, Zhao *et al* in 2013 isolated CPV-2a and performed the animal experiment. Nine beagles were inoculated with $10^{5.86}$ of 50% tissue culture infectious doses ($TCID_{50}$) of the virus. The following results were observed, all the experimentally infected beagles exhibited mild to moderate mucoid or watery diarrhea on day 4 post-infection. On day 9 p.i., characteristic histopathological lesions were observed in multiple organs of infected dogs, including liver, spleen, kidney, brain and all segments of the small and large intestines, while viral DNA and antigen staining could be detected in the sampled tissues. The titre of the isolated virus was determined by the method of Reed and Muench and was calculated to be $10^{4.86}$ $TCID_{50}$/100 µl.

4.2.5 Cross Neutralisation Test

For the cross neutralization test virus was kept constant and dilutions of serum were made. Therefore, 100 TCID50 of the virus was used for both the virus types i.e. CPV 2a and CPV 2b. The two fold serial dilution of the hyper immune serum produced against both the CPV types in rabbits were made and used in neutralization test.

Table 15: Depicting the maximum dilution of serum at which it can neutralize the CPV

Sets of serum-virus mixture	Two fold serial dilutions of serum													
	1:2	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**	**1:256**	**1:512**	**1:1024**	**1:2048**	**1:4096**	**1:8192**	**1:16384**
SET I	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	CPE observed
SET II	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	CPE observed	CPE observed
SET III	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	CPE observed	CPE observed	CPE observed
SET IV	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	No CPE	CPE observed

For cross neutralisation test, we formed four serum-virus groups with each group including various dilutions of serum. The plates were observed daily for the development of cytopathic effects. The results were noted when CPE was observed in the virus control for each set of serum-virus (Table 15).

Thus the maximum dilution at which serum against CPV 2a could neutralize CPV 2b virus is 1:4096 and the maximum dilution at which serum against CPV 2b could neutralize CPV 2a is 1:2048 (Table 16). The titre of the serum is the reciprocal of the dilution of the serum. Therefore the titre of serum 2a at which it can neutralize virus CPV 2b is 4096 and the titre of the serum 2b at which it can neutralize virus CPV 2a is 2048. The titre of both the serum for the homologous virus type is higher i.e. 8192.

Table 16: Titre of serum at which it neutralizes the virus completely

	SET I	SET II	SET III	SET IV
SERUM	2a	2a	2b	2b
VIRUS	2a	2b	2a	2b
Titre of serum	8192	4096	2048	8192

In an experiment conducted by Teramoto *et al* in 1995 to compare Enzyme-Linked Immunosorbent Assay, DNA Hybridization, Hemagglutination, and Electron Microscopy for detection of Canine Parvovirus infections, neutralization titers were also determined in the procedure by incubating antibodies diluted in minimal essential medium with 100 to 500 50% tissue culture infective doses of parvovirus. The neutralization titer was expressed as the highest dilution of antibody at which no infected cells were detected.

Reitzenstein *et al* in 2012 assessed the serological response to vaccination by quantification of CPV neutralizing antibodies. The neutralization titre was determined in a micro-titre serum neutralization test for the detection of neutralizing antibodies to CPV-2. The sera were tested in a SN test using dog kidney (DK) cells and 50 to 300 $TCID_{50}$ of the CPV-2 as the reference virus. SN titers were calculated using the Spearman-Karber formula.

In a study by Gamoh *et al* in 2005, an FPLV-based vaccine was able to cross-protect against a challenge with a virulent CPV-2b strain. According to Parrish *et al*

(1991) and Nakamura *et al* (2001a) using cross-neutralisation studies, the antigenic differences between FPLV vaccines andCPV-2 variants appear to be much more marked, reflecting the number of mutations scattered throughout the VP2 protein (Decaro *et al* 2010).

The antigenic relationships among the original canine parvovirus type 2 (CPV-2) and the variants CPV-2a, -2b, and -2c were evaluated. Cross-antigenic evaluation revealed clear differences among the CPV variants, which were appreciable by serum neutralization (SN). Antigenic differences were found mostly between the original CPV-2 and the variants, but they were also observed among the variants CPV-2a, -2b, and -2c. (Cavalli *et al* 2008).

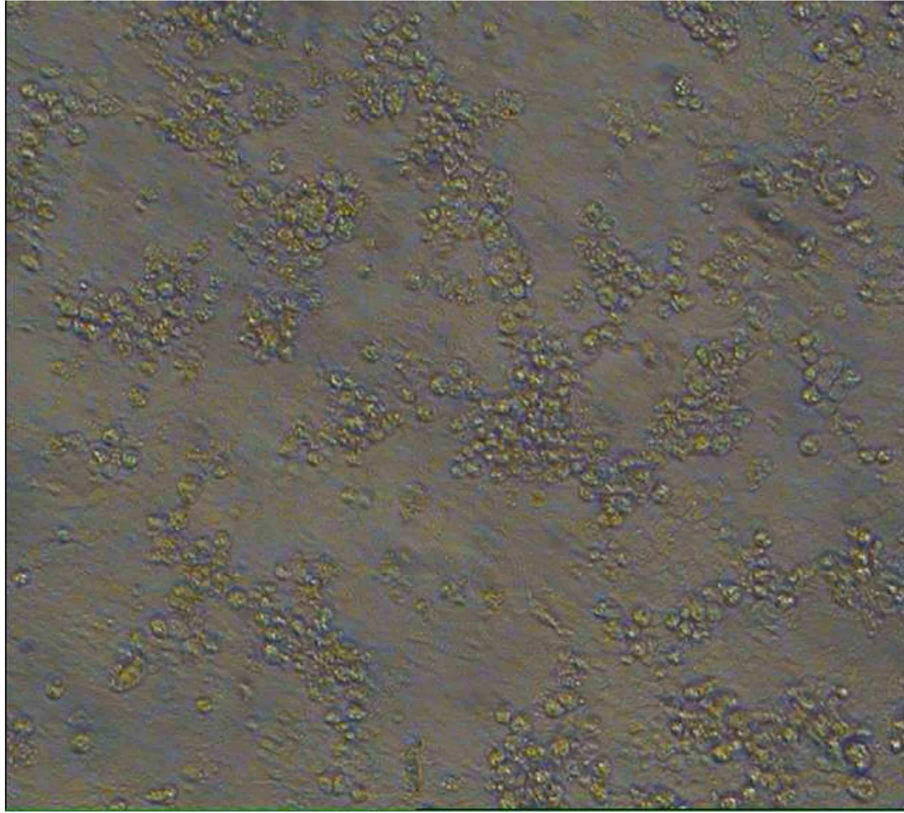


Fig. 19: Virus Control depicting CPE for virus CPV 2a

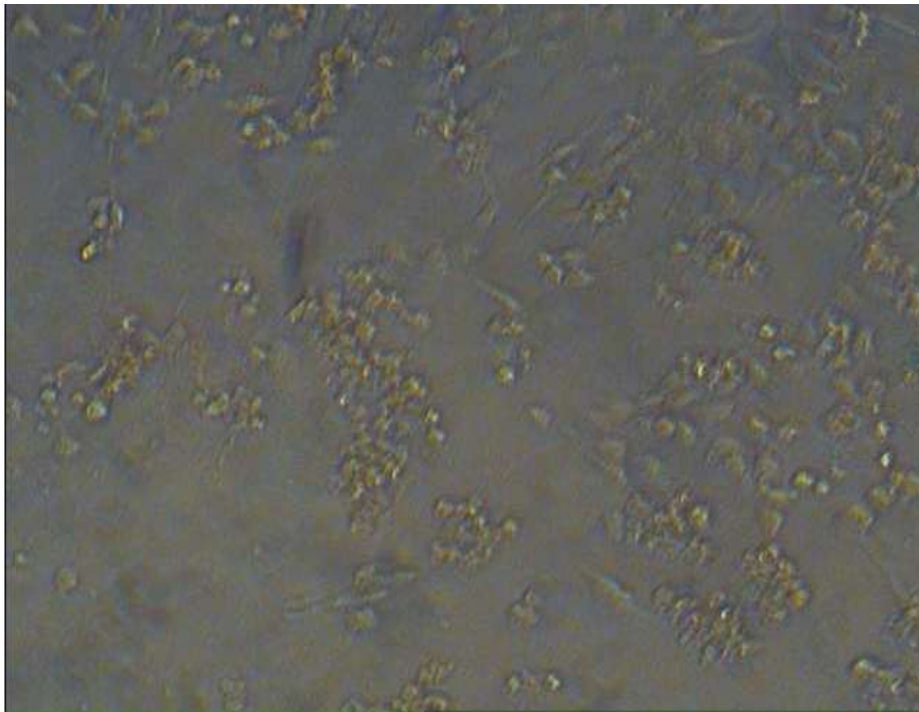


Fig. 20: Result for set II serum 2a and virus 2b (1:8192)

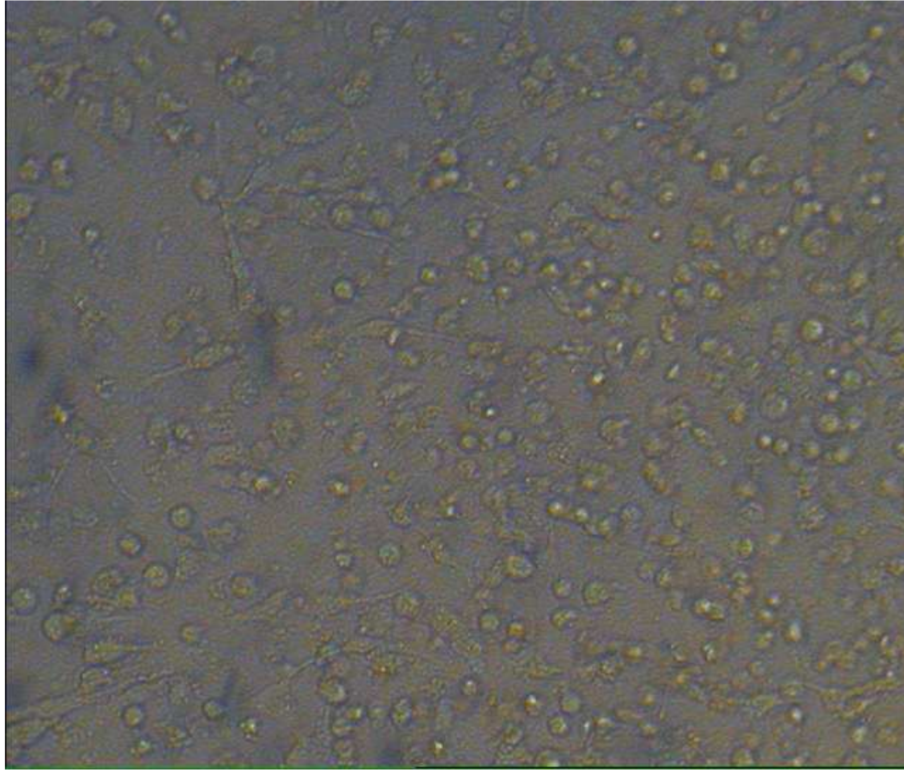


Fig. 21: Virus Control depicting CPE for virus CPV 2b

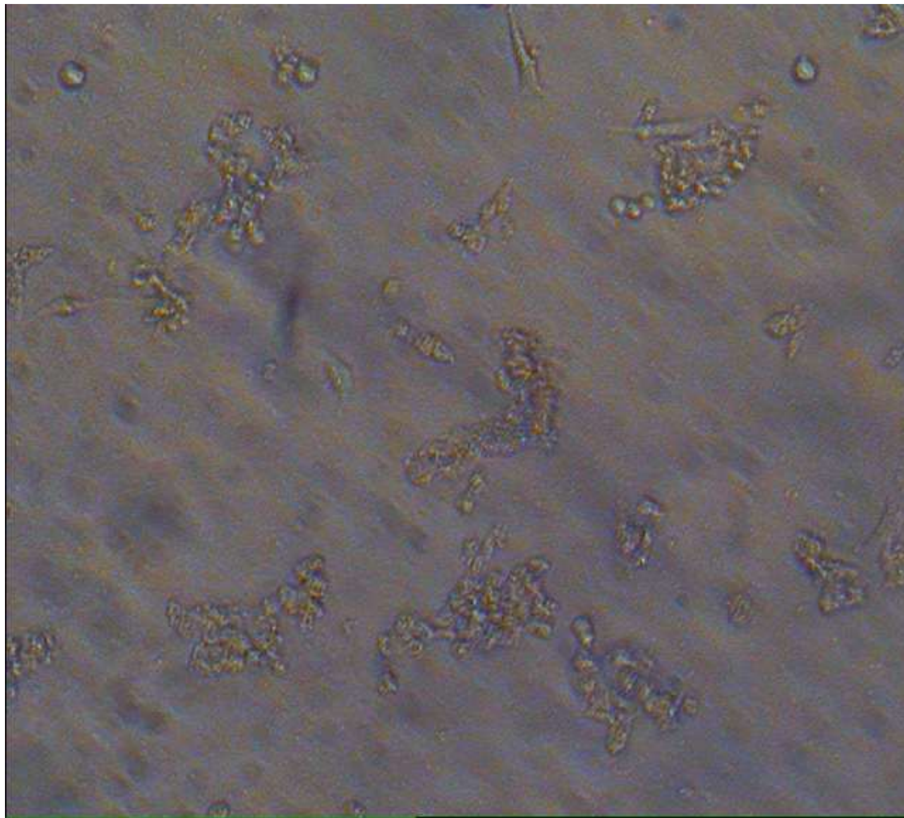


Fig. 22: Result for set III serum 2b and virus 2a (1: 4096)

CHAPTER V

SUMMARY AND CONCLUSIONS

Canine Parvovirus (CPV), a contagious disease mainly affecting dogs, is caused by a small, non-enveloped, single stranded negative sense DNA virus, 5.2kb in length. Analysis of isolates collected since its emergence revealed that viruses circulating after 1980 were antigenically different from earlier isolates. The antigenic variants of CPV were identified by several countries using monoclonal antibodies and were termed as CPV 2, CPV 2a, CPV 2b and CPV 2c.

A Real Time PCR was conducted on a total of 178 samples collected from Punjab, Assam, J&K, Chandigarh and Delhi to assess number of positive samples for each CPV 2, CPV 2a and CPV 2b. Out of the total 178 samples, 27 were positive for CPV 2, 86 were positive for CPV 2a and 20 samples for CPV 2b. Another interesting fact discovered was that a few samples were positive for more than one CPV variant simultaneously.

To study the whether the antigenic types of CPV cross protect each other, cross neutralization assay was performed for two types of CPV. For this study CPV 2a and CPV 2b were produced in bulk in MDCK cell line, cytopathic changes were observed and virus was harvested from the inoculated cell line. The cell culture supernatant was confirmed by PCR and nested PCR and the band for both CPV 2a and CPV 2b isolate was observed at 1198bp and 548bp respectively.

The bulk virus was purified by ultra centrifugation at 1,00,000g for 2 hours and the protein concentration of the purified virus was estimated by nanodrop method and it was found to be 93µg/ml for CPV 2a and 531µg/ml for CPV 2b. The purified virus of both the CPV types (CPV 2a and CPV 2b) was used to raise hyper immune serum in rabbits which was finally collected by intra cardiac route. The antibody titre was estimated by Indirect ELISA and was found to be 99606.24 and 86940.95 for CPV 2a and CPV 2b respectively.

The $TCID_{50}$ of the purified virus was calculated by Reed and Muench formula and was calculated as 10^3/ml for CPV 2a and $10^{2.53}$/ml for CPV 2b. Four sets of serum-virus mixture were made for cross-neutralisation tests in which two fold serial dilution of serum and 100 TCID50 of each virus was used. The following results were

obtained. The maximum dilution at which serum against CPV 2a could neutralize CPV 2b virus was 1:4096 and the maximum dilution at which serum against CPV 2b could neutralize CPV 2a was 1:2048. The titre of the serum is calculated as the reciprocal of the dilution of the serum. Therefore the titre of serum 2a at which it can neutralize virus CPV 2b was 4096 and the titre of the serum 2b at which it could neutralize virus CPV 2a was 2048. The titre of both the serum for the homologous virus type was found to be higher than former i.e. 8192.

CONCLUSIONS

1. CPV 2a was found to be most prevalent in the regions of India under study.
2. Samples were also found to be positive for more than one antigenic type of CPV simultaneously.
3. The Canine Parvovirus was successfully isolated in MDCK cell line.
4. Hyperimmune serum was successfully raised in rabbits confirmed by Indirect ELISA.
5. $TCID_{50}$ of the virus was found to be 10^{3}/ml for CPV 2a and $10^{2.53}$/ml for CPV 2b.
6. The maximum dilution at which serum against CPV 2a could neutralise CPV 2b virus was 1:4096 and the dilution at which serum against CPV 2b could neutralise CPV 2a virus was 1:2048.
7. The titre of serum 2a at which it can neutralize virus CPV 2b was 4096 and the titre of the serum 2b at which it could neutralize virus CPV 2a was 2048.
8. The titre of both the serum for the homologous virus type was found to be higher 8192.

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