MOLECULAR CHARACTERIZATION OF CANINE PARVOVIRUS-2 INFECTION IN AND AROUND NAGPUR.

T H E S I S

Submitted

in partial fulfillment of the requirements for the Degree of

MASTER OF VETERINARY SCIENCE

IN

VETERINARY MICROBIOLOGY

BY

DORLIKAR PAYAL RAJESH

Enrolment No. V/11/095

Nagpur Veterinary College, Nagpur-440006

MAHARASHTRA ANIMAL AND FISHERY SCIENCES UNIVERSITY,NAGPUR-440006

(INDIA)

2018

DECLARATION OF STUDENT

I hereby declare that the experimental research work and interpretation of the thesis entitled **"MOLECULAR CHARACTERIZATION OF CANINE PARVOVIRUS-2 INFECTION IN AND AROUND NAGPUR."** or part thereof has not been submitted for any other degree or diploma of any university, nor the data have been derived from any thesis/publication of any University or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

Date:

Place: Nagpur

(**DORLIKAR PAYAL RAJESH)**

Enrolment No. V/11/095

(Dr.S.R.Warke)
Chairman, Advisory Committee
Assistant Professor
Department Of Veterinary Microbiology
and Animal Biotechnology T& R Cell.

DECLARATION OF ADVISORY COMMITTEE

DORLIKAR PAYAL RAJESH has satisfactorily prosecuted her course of research for a period of not less than one semester and that the thesis entitled, **"MOLECULAR CHARACTERIZATION OF CANINE PARVOVIRUS-2 INFECTION IN AND AROUND NAGPUR."** submitted by her is the result of research work and is sufficient to warrant its presentation to the examination in the subject of **VETERINARY MICROBIOLOGY** for the award of **Master of Veterinary Science** degree by the Maharashtra Animal and Fishery Sciences University, Nagpur.

We also certify that the thesis or part thereof has not been previously submitted by him for a degree of any other university.

Place: Nagpur

Date:

(Dr. S.R. Warke)
Advisor/Guide
Assistant Professor,
Department of Veterinary Microbiology and
Animal Biotechnology T& R Cell

Name and Designation of Advisory Committee

	Name	Designation	Signature
1.	**Dr. V.C. Ingle**	Associate Professor, Department of Veterinary Microbiology and Animal Biotechnology T & R Cell	____________
2.	**Dr. U.M. Tumlam**	Assistant Professor Department of Veterinary Microbiology and Animal Biotechnology T & R Cell	____________
3.	**Dr. P.A. Tembhurne**	Assistant Professor, Department of Veterinary Microbiology and Animal Biotechnology T & R Cell	____________
4.	**Dr. P.M. Sonkusle**	Assistant Professor, Department of Veterinary Pathology	____________

CERTIFICATE

This is to certify that the thesis entitled, **"MOLECULAR CHARACTERIZATION OF CANINE PARVOVIRUS-2 INFECTION IN AND AROUND NAGPUR."** submitted by **MISS PAYAL RAJESH DORLIKAR** to the Maharashtra Animal and Fishery Sciences University in partial fulfillment of the requirement for the degree of **Master of Veterinary Science** in the subject of **Veterinary Microbiology** has been approved by the Student's Advisory Committee after examination in collaboration with the External Examiner.

Name & Signature of External Examiner	Signature with Seal Head of Department	Name & Signature Advisor/Guide Designation

Advisory Committee

	Name	Designation	Signature
1.	**Dr. V.C. Ingle**	Associate Professor, Department of Veterinary Microbiology and Animal Biotechnology T & R Cell	____________
2.	**Dr. U.M. Tumlam**	Assistant Professor Department of Veterinary Microbiology and Animal Biotechnology T & R Cell	____________
3.	**Dr. P.A. Tembhurne**	Assistant Professor, Department of Veterinary Microbiology and Animal Biotechnology T & R Cell	____________
4.	**Dr. P.M. Sonkusle**	Assistant Professor, Department of Veterinary Pathology	____________

Associate Dean
Nagpur Veterinary College,
Nagpur

Acknowledgement

Writing this thesis has been fascinating and extremely rewarding. I would like to take some time to thank all the people without whom this thesis would never have been possible. Many people have contributed to the research in their own particular way and for that I want to give them special thanks.

*To commence with, I pay my obeisance to GOD, the almighty to have bestowed upon me good health, courage, inspiration, zeal and the light. After GOD, first and foremost, I express my esteemed and profound sense of gratitude to my respected and enthusiastic research guide, my mentor,**Dr.S.R.Warke** ,Assistant Professor,Department of Veterinary Microbiology and Animal Biotechnology Teaching and Research Cell,Nagpur Veterinary College ,Nagpur,MAFSU,Nagpur for Her expertise, Sweetness,invaluable guidance, constant encouragement, affectionate attitude, understanding, patience and healthy criticism added considerably to my experience. Without her continual inspiration, it would have not been possible to complete this study.*

*I would express my sincere thanks to **Dr.A.S.Bannalikar**,Associate Dean,Nagpur Veterinary College ,Nagpur for providing all the facilities for the conduction of this research work.*

*With respect I owe my special thanks to **Dr.V.C Ingle**,Associate Professor, Department Of Veterinary Microbiology And Animal Biotechnology Teaching And Research Cell,Nagpur Veterinary College ,Nagpur,MAFSU,Nagpur for his kindness and constant encouragement in my research work and throughout my post graduate study.*

*I take this opportunity to express my deepest sense of gratitude and heartfelt obligation to **Dr.U.M.Tumlam**, Assistant Professor, Department Of Veterinary Microbiology And Animal Biotechnology Teaching And Research Cell,Nagpur Veterinary College ,Nagpur,MAFSU,Nagpur for her kindness,understanding,heart-warming support and noble guidance throughout my post graduation study and in my research work.*

*It is my privilege to express my thanks to **Dr.P.A Tembhurne** , Assistant Professor, Department Of Veterinary Microbiology And Animal Biotechnology Teaching And Research Cell, for his valuable suggestions ,scholarly guidance, which served as a constant source of inspiration throughout my study and research work. It gives me immense pleasuring thanking **Dr P.M Sonkusle**, Department Of Veterinary Pathology, for his ideas and constant inspiration. I am really grateful and thankful to **Dr.S.P.Awandkar**, Assistant Professor, and Department Of Veterinary Microbiology And Animal Biotechnology Teaching And Research Cell, Nagpur for his valuable ideas and constant inspiration and encouragement.*

I like to express thanks to ***Dr.J.A.Kesharkar sir*** *for their kind advice and constant encouragement throughout my post graduation study*

My acknowledgement would be incomplete if I would not record my thanks to my seniors ***Dr.Anand sir, Dr.Rucha mam,Dr.Sneha mam*** *and* ***Prashant sir*** *for his moral support, constant encouragement and kind advice throughout my post graduation study.*

*I cannot forget friends who went through hard times together, cheered me on,and celebrated each accomplishment, I sincerely admire the contribution,love,constant support of all my colleague,****Dr.Manoj Ade Sir,Dr.Priyanka,Dr Preeti, Dr.Pooja****,for their kind ,sweet co-operation and unstinted support, timely motivation, sympathetic attitude and unfailing help during the course of entire study and research work..I am also thankful to my lovely juniors* ***Dr.Sunny, Dr.Shruthy, Dr.Pragati, and Dr.Gauri*** *for their constant and wholehearted support.*

I would like to thanks by friends who always support me ,cheered me in any situation ,thanks to ***Dr.Aakansha, Dr.Noopur, Dr.Pankaj, Dr.Dushant,Dr.Pratik sir, Dr.Gauri, Dr.Sneha, Dr.Anjali, Dr.Ushma di, Dr.Juhi*** *for their support during whole study.*

I would like to special thanks to ***Bhujade kaka*** *for their continuous assistance in my research work.*

I thank god for giving me such a good friends ***Manu, Pratik, Ankita*** *who always support, loved, cheered, helped me whenever I required.*

Words prove a meagre media to write down my feelings for my life, my ***Sweet Parents (Rajesh Dorlikar and Radha Dorlikar)*** *and beloved brother* ***Shreyash*** *for their unconditional trust, timely encouragement, divine presence and supreme power the almightily without their blessing I wouldn't have been here.*

Date:
Place: Nagpur — Grateful and indebted

Payal Rajesh Dorlikar

TABLE OF CONTENTS

SR NO.	CHAPTERS	*PAGE NO.*
1.	INTRODUCTION	1-4
2.	REVIEW OF LITERATURE	5-22
3.	MATERIALS AND METHODS	23-37
4.	RESULTS AND DISCUSSION	38-56
5.	SUMMARY AND CONCLUSIONS	57-60
A)	BIBLIOGRAPHY	i-xv
B)	APPENDICES	xvi-xviii
C)	VITA	xix
D)	THESIS ABSTRACT	xx-xxii
E)	प्रबंध सारांश	xxiii-xxvi

LIST OF TABLES

Table No.	PARTICULARS	Page No.
1	Details of sample collection from in and around Nagpur	24
2	Details of Primers specific for VP2 gene of CPV used in PCR	29
3	Distribution of HA titres of CPV positive faecal samples with their frequencies	40
4	Details of Age, Sex, Breed and Vaccination Status of samples positive by HA	41
5	Details of Age, Sex, Breed and Vaccination Status of samples positive by Antigen Detection Kit.	44
6	Details of Age, Sex, Breed and Vaccination Status of samples positive by PCR.	47
7	Details of detection of CPV by conventional and molecular diagnostic tests.	51
8	Sensitivity and Specificity of antigen detection kit in relation to PCR for detection of Canine Parvovirus Antigen.	52

LIST OF FIGURES

Fig. No.	PARTICULARS	After Page
1	Age-wise status of Canine Parvovirus by HA	42
2	Sex-Wise distribution of Canine Parvovirus by HA	42
3	Breed-Wise Prevalence of Canine Parvovirus by HA	42
4	Prevalence of Canine Parvovirus by HA based on the vaccination status.	42
5	Age-wise status of Canine Parvovirus by Antigen detection kit	45
6	Sex-Wise distribution of Canine Parvovirus by Antigen detection Kit	45
7	Breed-Wise Prevalence of Canine Parvovirus by Antigen detection kit	45
8	Prevalence of Canine Parvovirus by Antigen detection kit based on the vaccination status	45
9	Age-wise status of Canine Parvovirus by PCR	49
10	Sex-Wise distribution of Canine Parvovirus by PCR	49
11	Breed-Wise Prevalence of Canine Parvovirus by PCR	49
12	Prevalence of Canine Parvovirus by PCR based on the vaccination status	49
13	Comparison of CPV-2 positive samples by HA,Antigen detection kit and PCR.	51

LIST OF PLATES

Sr. No.	PARTICULARS	After Page
1	Dog showing Bloody diarrhea (CPV-2)	39
2	Dog showing Vomition (CPV-2)	39
3	Dog showing acute Bloody diarrhea (CPV-2)	39
4	Haemagglutination Test of CPV with vaccines and control with Pig RBCs	40
5	HaemagglutinationTest of CPV with Pig RBCs to samples	40
6	UbioquickVET Canine parvovirus antigen rapid test kit showing positive and negative reaction for CPV antigen.	43
7	Samples compare with commercially available Parvovirus vaccine.	43
8	Amplification of VP2 gene of Canine parvovirus-2 by PCR.	46
9	Phylogenetic analysis of VP2 gene of CPV-37 sample with vaccines, Indian and World isolates.	53
10	Monolayer of MDCK cell line at 10 x magnification	56
11	Rounding of cell start at 24 hours of post-infection at 10 x magnification	56
12	Complete rounding of cell after 48 hours of post-infection at 10 x magnification	56
13	Clumping of cell observed after 72 hours of post-infection at 10 x magnification	56
14	Detachment of cell observed after 96 hours of post-infection at 10 magnifications.	56

LIST OF ABBREVIATIONS

CPV	Canine Parvovirus
%	Percentage
ORF	Open Reading Frame
mRNA	Messenger RNA
PCR	Polymerase Chain Reaction
VP	Viral Protein
NS	Nonstructural Protein
RNA	Ribonucleic Acid
gm	Gram
WBC	White Blood Cells
PI	Post Infection
IP	Incubation Period
RFLP	Restriction Fragment Length Polymorphism
HA	Haemagglutination
bp	Base Pair
For	Forword
Rev	Reverse
CPE	Cytopathic Effect
CRFK	Crandell Reese Feline Kidney
Hr	Hours
^0C	Degree Celsius

NV	Non Vaccinated
V	Vaccinated
mm	Milimeter
OIE	Office International Des Epizooties
RBC	Red Blood Cells
RPM	Revolutions Per Minute
ml	Mililitre
$MgCl_2$	Magnisum Chloride
ug/ml	Microgram Per Mililitre
DDBJ	DNA Data Bank Of Japan
NCBI	National Center For Biotechnology Information
GM	Growth Medium
DMEM	Dulbecco's Modified Eagle's Medium
TPGV	Trypsin Phosphate Glucose Versin
ELISA	Enzyme Linked Immunosorbant Assay
MDCK	Madin Darby Canine Kidney Cell line
et al.	*Et alia*
M	Molar
V	Vaccinated
NV	Non vaccinated

INTRODUCTION

The bond between human and animal is a mutually favorable and the dynamic relationship which positively influence the health of both. Many animals and birds kept as a pet but dog have a great popularity in the world as a pet. They perhaps the closest from pets to sporting, hunting companions to service dog, bomb detecting squad and healer of both physical and emotional problems of humans, perceive criminals and guiding blind people. Canine takes the valuable degree of various qualities to the worlds of the human; they share their lives with them, such strong bond between the dog owners with their pet have brought to great concern about their health and well being. In view to need have dog in various aspects, it is necessary to study the various diseases of dog which caused high morbidity and case fatality rate.

Dog get infected with various infectious agents, in which viruses of various families affect the dog health and cause heavy mortality. In India according to 2012, there were 11.2 million pet dogs and 17.1 million stray dogs. Among the animal diseases mostly viral enteritis in dog has become an important problem throughout the world.

Diarrhoea in dog is a major occurrence with myriads if not treated on time then it could have fatal outcomes. Certain virus like canine parvovirus -2, rotavirus, canine distemper, canine corona virus has been pinpointed as a major cause of viral gastroenteritis in dog. Among all viral disease, canine parvovirus-2 is a leading source of diarrhea in dogs under 6 month of age. (Schulz *et al.*, 2006).

Canine parvovirus -2 is a highly infectious viral disease of dogs and one of the serious concerns to pet owners, practicing veterinarians and scientists due to its high morbidity and mortality rate.CPV-2 is stable, highly contagious virus which mainly affecting canine population.CPV-2 spread by direct or indirect contact with infected faeces of dogs. Vaccine against canine parvovirus prevents this infection but case fatality can reach up to 91% if cases are untreated.

During late 1970s, a new contagious viral disease of pups emerged showing gastroenteritis or myocarditis like symptoms throughout the world, named as CPV-2.It was first recognized in 1977 and described in several

countries in mid 1978, then it has been established as a whole pathogen of dogs throughout the world. After a time of adaptation, the virus begin to be highly contagious for dogs, resulting in the prevalent and apparent in 1978–1979.CPV-2 virus has 98% similarity with *feline panleukopenia* , only differ in two amino acids present in the viral capsids proteins VP2 and also homogeneous to Mink enteritis and parvovirus of Foxes and Raccoons.

Canine parvovirus belongs to family *Parvoviridae* comprising two subfamilies *Parvovirinae* and *Densovirinae*. *Parvovirinae* contain virus of vertebrates and *Densovirinae* contain virus of insects. There are 3 genera in sub family *Parvovirinae*viz, *Parvovirus Erythrovirus* and *Dependovirus*. (Murphy *et.al*.,1999). Sequence analysis has also divulged that CPV strains have undergone a series of evolutionary selections in nature, which was globally distributed as new variants which have completely replaced the original CPV-2. Recently, CPV has three main antigenic variants viz. 2a, 2b and 2c which are distributed among the dog population throughout the world. (Decaro *et al*.,2006). Previously, CPV-2c was nominated as CPVGlu-426 mutant that originated initially from Italy, but now has been detected in other countries also (Buonavoglia *et al*., 2001). The CPV-2 has spread in identical pattern and the disclosure and subsequent renewal by the new antigenic strains has also been shown by antigenic analysis of isolates from Germany, France, Denmark, Japan, Italy, Australia, Spain and Africa although the proportions of CPV-2a, CPV-2b and CPV-2c strains has been differ. (Parrish *et al*.,1988, Mochizuki *et al*.,1993, Ybanez *et al*.,1995, Truyen *et al*.,1996, Steinel *et al*.,1998, Buonavoglia *et al*., 2001). These viruses have become distributed globally and there antigenic as well as genetic variation in the virus strains have also been corresponds with changes occurs in host range of the virus (Parrish *et al*., 1999). Phylogenetic analysis has divulged that CPV strains have undergone a sequence of progressive selections in nature, which have resulted in worldwide distribution of current variants have been replaced the original CPV-2.

Canine parvovirus-2 are non enveloped having 25nm in diameter with icosahedral symmetry. Genome consist of single stranded negative sense DNA virus having size of 5.2 kb in length (Murphy *et al*.,1999). Terminal palindromic sequences at each end to form hairpin structure.CPV-2 genome has 2 open reading frame (ORF) which are further divided into ORF 1 and ORF 2.The ORF1

encodes for two nonstructural protein NS1 and NS2 that are translated through alternate splicing of the viral mRNAs and ORF 2 encodes for two capsid protein i.e. VP1 and VP2.

The disease is widespread prevalent in Asia (Khadilkar *et al.*, 1994). In India, prevalence of canine parvovirus was first announced by Balu and Thangaraj in 1981 in Madras and Ramadas and Khader in 1982, confirmed that CPV-2 is an etiological agent of Canine parvovirus infection. After that enormous number of outbreaks of Canine parvovirus have been noted in various clinic from various empire of India like Kerala, Tamil Nadu, Karnataka, Andhra Pradesh, Odisha,Pondicherry (Mukhopadhyay *et al.*,2014), Haryana (Savi *et al.*,2009) and Uttar Pradesh (Nandi *et al.*,2010, 2010a).

The virus is transmitted via oro-fecal route and requires rapidly dividing cells of fetuses and new born or of hematopoietic and intestinal tissues of young and adults animals. (Decaro and Buonavoglia., 2012).

VP2 which is a major capsid protein of CPV, play a major role in the diagnosis of host range and antigenicity of CPV. Mutation which affect VP2 gene are predominantly responsible for evolving different antigenic variant of CPV-2.(Phromnoi *et al.,*2010,Kaur *et al.*,2015).The constant emergence of new strain of CPV-2 is a big concern of dog owners, veterinarian around the world.

Combined vaccine of CPV contains an inactivated and modified virus. These vaccine help to prevent CPV, but when there is emergence of newer antigenic variant, vaccine get fail to protect pups against CPV, so identification along with the knowledge of genetic variation of VP2 can be help in detecting the new strain of CPV and it help in development of new vaccine.

Diagnosis of disease have been done by using various methods like Haemagglutination (HA) Haemagglutination inhibition test (HI) ,Rapid antigen detection kit, agar gel precipitation test (AGPT),Enzyme linked immunosorbent assay (ELISA),electron microscopy, indirect florescent antibody test, virus isolation by cell culture using Madin darby canine kidney (MDCK),Crandle feline kidney (CRFK) and A-72 cell lines. Among them, virus isolation done by using various cell lines is highly specific way to identification, HA and HI are simple, conventional method of diagnosis. For electron microscopy, concentrated virus to be required. ELISA using monoclonal antibodies is also the another way for

diagnosis of CPV.Restriction Enzyme (RE) digestion, real time PCR, Loop-mediated isothermal amplification (LAMP), Nucleic acid hybridization or dot blot, in situ hybridization, nucleic acid sequencing .Among all conventional method, molecular assay like PCR ,nested PCR are highly specific and sensitive way of diagnosis. Real time PCR is also sensitive and specific technique for parvoviral DNA.

Currently, it has been observed that the canine parvovirus serotypes circulating in the Indian landmass is completely different from that included in the vaccine and may leading to decrease in the efficacy of the CPV-2 build vaccines. (Greenwood *et al* 1995., Yule *et al* 1997, Pratelli *et al*., 2001) could be the one reason for the infection in the vaccinated pups (Savi *et al*., 2009). Vaccine which are used in India against canine parvovirus is inactivated, modified live vaccine which protect the pups, but recent study observed that the canine parvovirus circulating in India is entirely different from strain included in the vaccine, so there is failure of vaccination observed, because some of the study noticed that, proportion of parvoviral infection in vaccinated and un vaccinated dog is nearly same, these may be the reason for the infection in the vaccinated pups.(Savi *et al.*, 2009).Considering this problem, it was observed strain used in vaccine that were isolated about 25 to 30 year ago (Nandi *et al*., 2010).

Most of the stray dogs are unvaccinated against the disease and they become the origin of disease to others susceptible host, early diagnosis of CPV infection by using conventional as well as molecular methods help to protect dogs.

Keeping in view of the above facts, the present study was aimed with following objective.

1. To study incidence of Parvoviruses among canines using conventional methods.
2. To characterize the Canine Parvovirus using molecular tools.
3. To establish the phylogenetic relationship of Parvoviruses based on VP2 gene sequence.

REVIEW OF LITERATURE

In a recent time, Canine parvovirus-2 reported to the leading causative agent of morbidity in dogs and that leads to great concern about health of dogs and their owners. In India, its (Canine parvovirus-2) prevalence is on higher side and even reported in vaccinated breeds. Taken into consideration the given study was undertaken for molecular characterization of Canine parvovirus in number of literature has been reviewed.

2.1 History of Canine parvovirus-2

As early as in 1970, Binn *et al.*(1970) reported the first evidence of virus and termed it as "Minute virus of Canine" (MVC) or CPV type 1 or CPV-1 when investigated in faecal samples of number of dogs.

Later on, Appel *et al.* (1978) reported a new type of dog disease with symptoms of severe gastroenteritis and myocarditis especially in pups, and it was first time noticed in USA in 1978, then it named as canine parvovirus type 2 and it was remained distinguished from other minute virus of canines that is Canine parvovirus-1.

According to Truyen *et al.* (1996) CPV-2 reported with number of nucleotide changes as compared to original CPV-2. These changes are reported to be at positions like 3045, 3685 and 3699 and for CPV-2b one more change was recorded at position 4026 and with these changes both CPV-2a and CPV-2b remain prominent world-wide and replace original CPV-2 in the given time.

According to Buonvoglia *et al.* (2001) new variant with antigenic change at a position with amino acid substitution Asp426Glu was recorded. This change leads to change in capsid protein which does act as antigenic moiety of CPV-2.

2.2 Classification of CPV-2:

Tattersall and Cotmore. (1990) while working on CPV-2 classification put forward that virus under family *Parvoviridae,*it remains classified with presence of subfamilies *Parvovirinae* and *Densovirinae.* The subfamily *Parvovirinae* generally infects vertebrates and again remained classified into three genera such as *Parvovirus, Erythrovirus* and *Dependovirus* and another

subfamily,*Densovirinae* able to infects insects also which remain classified into three genera such as *Densovirus, Iteravirus* and *Contavirus.*

Jones *et al.* (1997) hinted a close relation of Mink enteritis virus (MEV), Bluefox parvovirus (BFPV) and Raccoon Parvovirus (RPV) with CPV.

Buonavoglia *et al.* (2001) observed a variant of CPV-2 as mutant CPV-2C in dogs with amino acid change at 426th position from Asp to Glu.

Sakulwira *et al.* (2001) reported the time range when different CPV are originated. As per them, CPV being a member of autonomous *parvovirus* with single-stranded DNA of approximately 5Kb with non-enveloped structure reported first variant in 1979 as CPV-2, which was variant of CPV type 2a (CPV-2a). It remains widely spread and then again reported with another variant in 1984 as CPV-2b. In coming time, CPV-2b found to be replacing CPV-2a.

As per Shackelton *et al.* (2005) statement, viral evolution simply remains associated with emergence of Carnivore parvovirus.They proposed a theory that CPV-2 might have emerged some 10 years earlier before it was discovered as a disease. They proposed that with number of mutations, virus might have emerged as a new type from unknown source and produce a capability to infect new host and associated ability to spread. With the promising adaptation virus remained highly infective in dogs as evident in 1978-79. In a study, parvovirus when investigated in CPV-2 vaccine programme rapidly accumulated mutation reported *in vivo* in number of isolates and resultant virus found to be accumulating rapidly during the process of passage in tissue culture.

Carter and Wise. (2006) by doing comparative sequence analysis of *Feline panleukopenia* and CPV-2 virus for capsid proteinVP2, about 98% similarities was recorded indicated its close evolution.

Decaro *et al.* (2013) investigated the predominance of virus variants in which, CPV-2a remained most predominant in number of countries followed by CPV-2C and CPV-2b.

2.3 Virus Structure:

Parrish *et al.* (1991) stated Canine parvovirus evolution with number of antigenic type replacement and several sequence evolution. They found that

Parvovirus having 5000 bases of ssDNA able to encode two structural proteins i.e., VP1 and VP2 and non-structural protein i.e. NS1 and NS2. Here VP1 and VP2 are the resultant of alternative splicing of messenger RNA and they share high percentage of amino acid sequence homology. As a whole, X-ray structure of CPV when determined by X-ray crystallography about 60 -70 copies of VP2 proteins and 10 copies of VP1 found, it built up the capsid structure.

Tsao *et al.* (1991) reported VP2 monomer with 8 stranded antiparallel β-barrel with large loops remained inserted among the β-strands which can make up the capsid. Capsid surface remained with characteristic prominent region covered with three-fold axis of symmetry, with a circular canyon covering about five-fold axis of symmetry and remained with a depression of the two-fold axis of symmetry which interprets antigenic epitopes and remained affected by residue on the three-fold spike.

Strassheim *et al.* (1994) reported VP1 and VP2 are resultant of alternative splicing of messenger RNA from the viral DNA with a common sequence for the two proteins. Resultant gene is said at some time as VP1/VP2 gene. It is reported that proteins are comprised of 748 amino acids in both VP1 and VP2 proteins and becomes major component of non-enveloped icosahedral capsid of CPV-2 having 584 amino acid residues. Hardly any amino acid substitutions in its sequence can alter pertinent biological characteristics of the virus.

Lamas-Saiz *et al.* (1996) related the particular site (three sites) present on the capsid that can affect canine host range on the three fold spike which remained separated on 25 to 30 A^o. They reported that folding of flexibility of loops with capsid structure, associated with virus receptor interactions or in capsid uncoating. Here they reported CPV is having agglutination property of erythrocytes as co-evidenced in porcine, monkey and dog erythrocytes.

Weichert *et al.* (1998) reported similar to RNA virus, CPV-2 also remained variant in genomic content. Genome also found to be coding non-structural proteins, NS1 and NS2 along with structural proteins. NS1 simply remained covalently linked to 5’ end of newly synthesized DNA during packing and remained external to the capsid structure during later stages of infection. This present linkage reported to be disturbed by extracellular nucleases and finds special role in structural variation in capsid and in infection.

Murphy *et al*. (1999) prompted *Parvovirus*as non-enveloped, 25nm in diameter having icosahedral symmetry; they reported *Feline* and *Canine parvovirus* differences by involving mapped to the spike and found only two amino acid differences between the two isolates.

Mizak *et al.* (2001) suggested that VP3 is resultant of VP2 by post translational proteolytic removal of about 15 to 20 amino acids available at N-terminus and present only in complete virion. As per recording, amino acids sequence of VP2 and VP3 are readily available at C-terminus region of VP1. While amino acids of N-terminus region of VP2 found to be identical to all CPV isolate and host range variants such as cats, mink and raccoons.

2.4 Viral Replication:

Kasamatasu and Nakanishi. (1998) reported *Parvovirus* replicating in specific cells by involving cellular and viral determinants into consideration. Here *Parvovirus* prefers to grow by replication in nucleus of dividing cells. The virus simply gets attached to cell by binding to specific sialic acid having oligosaccharides moieties and uses them as receptors on the surface of the cell. Here virion gets entry into the cells and travels all the way to cytoplasm to reach nucleus by endocytic pathway by involving clathrin coated vesicles using the cells own nuclear import machinery and their after viral genome attempted entry into the nucleus via the nuclear pore complex.

Murphy *et al.* (1999) reported use of DNA polymerase to synthesize double stranded DNA via single stranded DNA viruses. Here 3' terminal hairpins are responsible for synthesis of double stranded circular DNA which remains as self-primer for synthesis. This dimeric form of replicative intermediates from which two complete positive strands and two complete negative strands are generated by involving a complicated series of re-opening of circular forms which reinitiate replication at transiently formed hairpins, and remain repeated single strand endonuclease cleavages.

Buonavoglia *et al.* (2001) reported Canine parvovirus type 2 evolution in Italy along with mutation rate of CPV genome. They put forward as the parvovirus DNA getting replicated by host cell DNA polymerases, chances of replication errors are very low and hence mutational events affecting biological properties are reported to be on lower side.

Parker *et al.* (2001) confirm the CPV entry in the host cell by involving viral casid co-localized with transferrin in perpendicular vesicles of cells shortly after entry, dynamic depend and by Clatherin-Mediated endocytic pathway.

Badgett *et al.* (2002) related viral attenuation with evolutionary dynamics. They reported that an incidence of pattern of evolution remains consistent with recombination and not with discrete selective sweeps of specific mutations.

Hoelzer and Parrish. (2010) reported *Parvovirus* infectivity in actively dividing host cells. Among pups older than 4 weeks, virus able to replicate in the tissue like bone marrow, spleen, lymph nodes and the progenitor cells in the crypts of Lieberkunhin of the intestine. They reported infection of neonatal animals and fetuses generally occurred during maternal immunity absence. In those other tissues can also get infected and resultant virus replication recorded in myocardium and/or cerebellum, which leads to myocarditis in puppies and appearance of cerebellar hypoplasia in kittens. In few cases, infection in utero also been linked with abortion or generalized viral replication in number of fatal organs.

Majer-Dziedzic *et al.* (2011) realized the presence of variants in Canine parvovirus (CPV-2) strains by phylogenetic analysis. When investigated in Poland, they reported number of *Parvoviruse's* prevalence records in cats and availability of even single mutation linked with basic biological trait change.

Decaro and Buonavoglia. (2012) summarized the available replication of Canine parvovirustakes place in nuclei of cell and mostly in dividing cells of intestinal tissues of young and adult animals, foetuses and even new-borns.

Srinivas *et al.* (2013) related replication of Canine parvoviruswith cell stage as S phase or early G2 phase of cell division cycle. This type of viral replication linked with profound pathogenesis of Canine parvovirus infection.

2.5 Canine parvovirus type 2 variants:

Binn *et al.* (1980) discriminated Canine parvovirus-2 from early parvovirus of dog that is minute virus of canines and referred as CPV-2. It is available worldwide as CPV-2 and reported to be endemic in many countries in domestic as well as wild dog population which leads to increased morbidity and mortality.

Buonavoglia *et al.* (2001) reported amino acid change in structural protein of type 2a and of CPV-2b as a variant Val instead of Ile. In Italy, they reported new strain of CPV-2 having amino acid substitution of aspartic acid to glutamic acid at 426 position.

Decaro *et al.* (2005a) reported Single nucleotide protein of CPV type 2a and 2b in capsid protein gene sequence which leads to determines antigenic variation in viral capsid.

Nakamuro *et al.* (2004) reported that antigenic features changes with mutation at 3' fold spike of the CPV-2 capsid. Based on mutation now antibodies can distinguish CPV type 2b and Glu-426 mutant CPV-2c.VP2 hypervariable region serves as a gene region of interest in characterisation of CPV-2.

Decaro *et al.* (2012) linked epidemiological and diagnostic aspects with two antigenic variants named as CPV-2a and CPV-2b when CPV-2 undergone genetic evolution. In recent time, CPV-2c was also detected in Italy and can spread rapidly to several countries.

2.6 Transmission of CPV-2.

Decaro *et al.* (2005b) put forward the transmission possibility of CPV-2 as a highly contagious infectious disease which can directly pass on to other dogs or via infected faeces. The dogs can easily transmit the infection into the environment, people or equipment. CPV-2 mainly getting transmitted by clothes, hand contact, shoes, food, water bowls; collars and leads to others.

Houston *et al.* (1996) stated that CPV is highly stable in the environment and can survive up to year. During severe infection more than 10^9 viral particles/grams of faeces able to shade in the faeces. This source reported to the deadliest source of infection. This virus also remained resistant to the heat,drought, cold and humidity.

Nandi *et al.* (2010) reported Canine parvovirus transmitted by oral contact with infected faeces as well as via contaminated surfaces. CPV simply can survive for months in faeces and can easily contaminated area for 5 months, if conditions are favourable. Dogs are also reported to be carrier for infected faeces as a source of contamination such as pet shops, breeding establishments, especially commercial set ups, veterinary hospitals and breeding kennels.

2.7 Clinical sign of CPV-2:

Stann *et al.* (1984) reported dog suffering from CPV infection observed with symptoms of bloody diarrhoea, anorexia, Leukopenia (WBC<6,000/mm^3) and fever (>39.6).

Meunier *et al.* (1985) conducted experimental study on sero negative Beagle dogs of age two to nine week old by infecting them with parvovirus inoculums via intravenous routs,found that more than 30 percent dogs with symptoms of pyrexia, vomiting, depression and diarrhoea.

Radostits *et al.* (2000) with the *parvovirus* infection, reported increased dehydration and reduction in elasticity of tissue. It is also linked with increased in body temperature which leads to hyperthermia and dehydration.

Joshi *et al.* (2001) examined CPV infected pups in early days of post inoculation as appearing mild dullness and anorexia and mild diarrhoea on 4th day of post inoculation. Upon 4 days, pups increased in infection resulted in shedding yellowish watery diarrhoea. In few cases it also started smelling diarrhoea with mucosal sheds upon 6th day of post inoculation. Smelling diarrhoea with mucosal sheds on day 6 of PI. On between 4 to 5 days of Post infection moderate rise in temperature was observed.

Gupta *et al.* (2006) reported dogs having symptoms like acute haemorrhagic enteritis and mycocarditis were detected by rapid PCR technique. Once dog get infected by Canine parvovirusthen they serve as source of infection for other healthy dogs by shedding virus in their faeces.

Nandi *et al.* (2010) confirmed the presence of Canine parvovirusin dogs by PCR and RE mapping of Dogs, where they appeared with vomition and haemorrhagic gastroenteritis in all age group. In addition few reported with myocarditis and heart failure in pups having age less than 3 months.

Decaro and Buonavoglia. (2012) reported CPV replicates in oral nasal route after inoculation especially in lymphoid tissue and infected leukocytes in 3-7 days of post inoculation in dogs. CPV get affinity to infecting leucocytes and induce lymphopenia.CPV able to replicate in cardiac cells of 2-3 weeks old sero negative pups causing fatal mycocarditis, level of haemorrhagic enteritis depend upon maternal antibody titre and time of getting infection. Initial clinical sign starts

with anorexia, depression, vomiting and bloody diarrhoea. Affection of pulmonary infection may leads to respiratory distress. When dogs are untreated them mortality reaches up to 70%.

Thomas *et al.* (2014) related 26% of dog's mortality with Canine parvovirus than all other viral diseases of dogs.

Mylonakis *et al.* (2016) clinical conditions were recorded with CPV-2 infection in host in the form of weakness, foul smelling diarrhoea, depression, anorexia which may form mucoid to purely hemorrhagic. It is also associated with dehydration and fever.

Kilian *et al.* (2018) reported dogs with chronic gastrointestinal problem once infected with CPV-2. It leads to increased cell turnover rate and replicate in intestinal crypt epithelium and lymphoid tissue.

2.8 Epidemiology of CPV2:

Hadi *et al.* (2011) investigated by PCR assay that Iranian isolates of CPV in faecal samples leads to high-level diarrhoea in 44 dogs. Among them, 39 dogs found to be positive for CPV-2b, 5 samples for CPV-2a, observed that the CPV-2b was predominant virus type. The presence of new antigenic variants, CPV-2a and CPV-2b were confirmed after identification of causative agent of CPV-2.

Mohyedini *et al*. (2013) suggested that earlier diagnosis by molecular and immune-chromatographic based found to be useful; since if dogs were detected earlier the mortality remains about 25-30% but it exceeds aggressively to about 91% if not detected earlier.

Pedro *et al*. (2014) linked molecular and serological surveillance for canine enteric viruses in dogs by processed 53 faecal samples, out of these 23 samples were positive for canine parvovirus and confirmed for the presence of 71.6% antibodies against Canine parvovirus. They found that unvaccinated dog population are increasingly getting infected by the CPV.

Parker *et al*. (2017) involved next generation sequencing for the study of 12 dogs' rectal swabs with symptoms of Parvovirus associated disease. They targeted RNA transcripts for better detection of only replicating virus of CPV.

Baba Sheikh *et al.* (2017) based on molecular concept and phylogeny, they investigated VP2 gene of Canines in Sulaimani Iraq and reported its world-wide distribution which remained fatal to the puppies. Sequenced PCR positive samples indicated that CPV-2 has a unstable DNA genome.

Oliveira *et al.* (2018) investigated Canine parvovirus 2c infection in dogs sampled from southern Brazil region. They found atrophy of villi, fusion of villi and diffuse segmental granulation. A mutating gene as VP2 was detected in number of isolates with 99.4 to 100% identity as per sequencing and diagnostic test.

Balu and Thangaraj. (1981) reported 305 CPV cases in India at Madras and Ramadass and Khader probably for the first time with the symptoms such as vomiting, diarrhoea, leucopenia and depression.

Savi *et al.* (2009) involved PCR and RFLP genotyping of field strains of Canine parvovirus from Haryana. They found all the fields isolates were CPV 2a and CPV 2b positive. In a study, use of RFLP to differentiate CPV-2 genetic variation found to be mainly important. They also reported the presence of CPV in vaccinated dogs; most possibly it may be because of vaccination failure.

Manoj Kumar *et al.* (2010) investigated Canine parvovirusat molecular level by involving cloning and restriction endonuclease analysis. They also involved PCR along with RE for successful targeting of CPV genome and reported proper management and jurisdiction of disease.

Parthiban *et al.* (2011) isolated Canine parvovirus from Puducherry,South India, by examine 128 faecal samples by HA ,ELISA,PCR and isolated in CRFK cell line. Typing of CPV-2 done by using monoclonal antibodies

Chinchkar *et al.* (2014) when investigated field Canine parvovirus by involving *in vitro* cross neutralization performed against vaccinated dogs sera they revealed that sera of original antigenic type CPV-2 found to be neutralizing antigenic variation 2a and 2b in a better manner. They proposed the statement that vaccine containing CPV type 2viruses can be the candidate to immunize the dog against prevalent CPV-2a and CPV-2b infection.

Thomas *et al.* (2014) based on PCR method detected epidemiological Canine parvoviral disease in dog based on PCR method in the region of Bareilly. They reported 52% samples of dog found to be positive for PCR.

Kaur *et al.* (2015) involved Madin Darby Canine Kidney (MDCK) cell line with PCR and nested PCR to detect the presence of CPV in the dogs. It was then sequenced then confirmed by sequencer for VP2 gene.

Kaur *et al.* (2015a) reported the prevalence of CPV-2 in dogs of Ludhiana, Punjab to about 11% by PCR and to about 50% by nested PCR. Result showed more sensitivity towards nested PCR.

Kulkarni. (2016) reported use of PCR-RFLP, to detect 9 sample positive for CPV-2 out of 25 samples tested for dog samples collected from Parbhani, Maharashtra. As per molecular phylogeny dominating CPV 2a is common in Parbhani.

2.9 Diagnostics of Canine parvovirus infection:

Disease diagnosis considered as a main step for cure of disease, as small outbreak disturb the whole population at a large scale directly or indirectly and hence better diagnostic facility ensures early control of the disease.

There are now several diagnostic methods are available for CPV detection in biological sample, such as molecular, serological and conventional.

Conventional method:

2.9.1 Detection of CPV-2 Antigen by Haemagglutination Test:

Scientist Al-Bayati *et al.* (2010) investigated from 90 sample size that, 66% of agglutinated RBCs and considered as clinically suspected pups. Titres value about 32 also recorded to be positive.

Chollam *et al.* (2012) compared PCR and HA test for CPV detection; among them with 76.11%, HA test scored better as only 54.87% positive cases recorded by PCR with the same sample. All PCR-positive samples were also positive by HA test. Hence HA test recorded to be the preliminary screening test over PCR and it remains low in cost makes it useful candidate.

Puentes *et al.* (2012) reported HA titer as 320 and 1280 in each sample of CPV-2c from symptomatic puppies and reported complete heamagglutination at highest dilution.

Silva *et al.* (2013) by testing 112 faecal samples of one year old puppies; test found HA positive with 32 samples with titres ≥128 and eight samples revealed low positive response with 32 and 64.

Chinchkar *et al.* (2014) by using 45 samples in HA test with Porcine RBCs; 23 samples found to be heamagglutinated and hence for identification of CPV, HA test recommended for sure.

Kulkarni. (2016) recorded titer >64 as positive for 9 sample out of 25 faecal samples by HA test. In porcine RBCs, this method reported to be handy, economic and easy to perform, for better diagnosis of CPV from faecal samples.

Muthuraj *et al.* (2016) investigated about 30 rectal swabs of dogs considering CPV infection. Results recorded that HA titre>32 was positive with9 samples (30%) and hence these dogs were reported diseased.

2.9.2 Detection of CPV-2 Antigen by Antigen detection kit:

Oh *et al.* (2006) succeeded in developing immuno-chromatography assay kit which detects antibodies of Canine parvovirus with 97.1% sensitivity and 76.6% specificity.

Schmitz *et al.* (2009) reported the 20% detection of CPV in acute haemorrhagic diarrhoea and chronic diarrhoea positive pups with 50 faecal samples by involving immuno-chromatography test.

Al- Bayati *et al.* (2010) done a comparative study between antigen detection kit and HA test with 90 faecal samples, in which antigen kit found to be more specific (98.8%) and sensitive (100%).

Reddy *et al.* (2012) used Scan Vet^{TM} PARVO kit to screen 30 random samples of diarrhoea dogs and found that 18 samples were positive.

Sundaran *et al.* (2015) reported success of immuno-chromatographic strip test when examined fifty faecal samples, among 18 samples were showed positivity and compared with PCR assay. Strip test showed 92.86% specificity as compared to 72.22% of PCR and hence recommended to use in diagnosis of CPV infections in dogs.

Apaa *et al*. (2016) were collected 53 samples from dogs, 64% tested positive for CPV-2 with the SNAP Parvo Antigen Test.

Hasan *et al.* (2016) reported the success of lateral flow assay when compared with PCR assay. They detected 20 samples found positive for CPV with (30.7%) prevalence out of 65 samples tested.

Folitse *et al.* (2018) established the presence of CPV infection in 61.11% samples tested by involving commercial rapid test kit which was based on immuno-chromatography principle.

Ahmed *et al.* (2018) while testing 40 rectal swab of diarrheic dogs by involving immuno-chromatography kit as FASTest PARVO strip; out of 40 samples 33 (82%) reported positive.

2.10 Detection of CPV-2 by Nucleic acid:

Polymerase chain reaction:

Kumar *et al.* (2011) investigated 52 faecal samples for CPV infection by involving nested PCR and its internal primer (PCPV-N set) and external primer. Among them, 27 samples reported positive with general PCR and n-PCR given 31 positive samples.

Parthiban *et al*. (2011) screened 128 rectal swabs for CPV-2 by using PCR, 18 samples which showed titre above 64 were found positive by PCR with 681 bp band.

Parthiban *et al.* (2012) by using differential PCR with 53 faecal samples, reported that CPV-2a and 2b antigenic type specific primers were able to detect 17 samples positive by CPV-2b specific primers.

Singh *et al.* (2013) recognized CPV by PCR when tested 100 dogs' faecal samples from Mathura, Uttar Pradesh, India. They identified 63% positive cases with no sex variation recorded with CPV prevalence and dogs of the age group of ≤6 months were prone to CPV infection in comparison to >6 months.

Srinivas *et al.* (2013) by working on molecular epidemiology principle able to detect CPV in southern India. By testing 128 samples of faecal type, primers set as pair H-for and H-rev able to detect 69 positive samples with 630 bp.

Chinchkar *et al.* (2014) while studying CPV isolates of India and its relevance with Canine parvovirus-2 vaccine; performed two independent PCRs and detected number of CPV-2 varieties and variants. Out of 45 samples tested by PCR by involving P2s/P2as and Pabs/Pabas primers, 27 samples found to be positive with 680 bp amplicon to confirm CPV presence in clinical samples

Kaur *et al.* (2015) recommended using PCR, in detection of CPV infection by using DNS molecules isolated from suspected species. They tested 100 samples; out of which 11 were found to positive for PCR yielding size of 1198 bp.

Behera *et al.* (2015) reported the cases of CPV infected dogs of Bhubaneswar, Odisha, in which 71 rectal swabs were found to be positive for 29 CPV cases as per PCR assay.

Apaa *et al.* (2016) collected 53 faecal samples of dogs having symptoms of gastroenteritis in Nigeria. When checked by PCR using two sets of primers for VP2 gene and one set for canine GAPDH, intensifying 430 and 193 bp.In a result about 20 samples found positive for VP2 gene by short primer of VP2.

Kulkarni*.* (2016) targeted 25 faecal DNA samples for CPV, check and among them 09 samples were found to be positive with a band of 747 bp.

Baba Sheikh *et al.* (2017) reported by molecular typing of PCR using P1 and P2 primers with size of 400 bp, samples were tested using the CPV-2ab primer pair expected size of 681 bp. CPV remained positive in dogs and further samples were screened by using specific primer CPV-2b.

Oliveira *et al.* (2018) tested 24 faecal samples of dogs in southern Brazil, out of which 17 samples found to be positive for PCR. These dogs were positive for symptoms like parvovirus enteritis.

Ahmed *et al.* (2018) by targeting VP2 gene with DNA sample of rectal swab of diarrheic dogs, out of tested 40 sample, about 33 samples reported positive for targeting with primer pair Hfor/Hrev.

2.11 Genome sequence analysis of CPV-2:

Nandi *et al.* (2015) targeted VP2 gene by PCR, Restriction endonucleases and DNA sequence analysis of 36 clinical samples of dog reported to be suffered from CPV. They sequenced further PCR positive

samples. Sequencing was done for six PCR positive samples, out of which three were distinguished as CPV-2c, designated that this CPV type 2c was currently circulating in India.

Decaro *et al.* (2013) by mass screening (615 faecal samples) of dogs in number of European countries by doing sequence analysis, dominance of CPV-2a were recorded in most of the countries, which was descendent by CPV-2C and CPV-2b.

Perez *et al.* (2014) by involving phylogenetic and genome sequencing of Canine parvovirus revealed field variants and emergence of a recombinant strains. They groups according to frequency,the higher-frequency bases ranged from 51.5 to 62.0% and belonged to a CPV-2c strain; the lower-frequency bases ranged in between 37.9 to 48.4% and belonged to a CPV-2a strain. They grouped Uruguayan CPV-2C and CPV-2a as two divergent phylogenetic groups and differentiated by both VP1/VP2 and nucleotide sequencing.

Kaur *et al.* (2015) were sequenced PCR positive samples using NCBI BLAST and analyzed using multiple sequence alignment software Clustal Omega, revealed that four samples P14, P44, P45 and P89 were showing homology with vaccine strains with 100% query coverage and 100% homology.Samples and vaccines were showed difference at the nucleotide position 2773, 2816, 2817, 2885, and 3189. From the multiple sequence alignment, it could be conclude that the two vaccines belonged to CPV-2 type and the further samples were associated to CPV-2b type.

Kulkarni.(2016) performed sequence analysis of nine PCR positive isolates,canine parvovirus sequenced contrast with , FPV from Argentina and USA , MEV from China, CPV-2 from USA, CPV-2a from India and China, CPV-2b from Spain, China and Italy and CPV-2c from Uruguay,USA, reportedthat the sequence variation among CPV-2b and CPV-2Cat a position 746 such as AAT (Asparagine) and Asp (Aspartic acid), respectively which showed its diversity with sequence homology. The nucleotide maximum divergence between isolates and other CPV-2a isolates from India is 0.84% and homology 99.16%.

Baba Sheikh *et al.* (2017) analysized total eight faecal samples for sequencing, Total Six virus sequences form clustered within the C5 branch, together with the CPV-2 from Egypt. Two remaining virus sequences clustered

within the C4 branch with CPV-2 sequences from Thailand, Taiwan, and China, terminated that CPV-2b strain was circulating in Sulaimani/Iraq.

La Torre *et al.* (2018) analysized total 35 stool samples from different puppies exhibiting signs of the disease and positives by PCR.Sequencing were analyzed with an ABI 3730 DNA Analyzer, succeeded in characterizing CPV-2a, CPV-2b and CPV-2c by targeting VP2 gene of infected domestic dogs in Eucador. They observed high prevalence of CPV-2a followed by CPV-2b and CPV-2c. In addition by sequence analysis number of amino acid substitution at position such as 101, 139, 219 and other at VP2 gene was reported.

Ahmed *et al.* (2018) by investigating 25 samples, reported the presence of CPV-2a with number of amino acid substitution such as Ser 297 Ala, Thr 390 Ala, and Asp 434 Gly and reported to be unique in its presence. The BLAST analysis manifested that the *feline panleukopenia virus* (FPV) sequences in samples which showed 99% sequence homology to other FPV sequences present in GenBank.

2.12 Phylogenetic analysis of Canine parvovirus 2:

Mohan Raj *et al.* (2010) involved phylogenetic analysis of canine *parvovirus* by proceeding ten clinical sample sequences, in a study it showed very close homology with Japan and India with one sequence while few of them remained in clade with CPV-2a strains of Korea, USA, Italy, Brazil, Germany, Taiwan and Vietnam and few sequences had distinct lineage but shared molecular relationship with new CPV-2a reference strains.

Majer-Dziedzic *et al.* (2011) constructed dendrogram tree by using the program POPGEN32 according to Nei's coefficient, and then the UPGMA algorithm (Unweighted Pair-Group Method Using Arithmetic Averages) was selected for hierarchical clustering analysis. as per phylogenetic analysis strains of Poland revealed phylogenetic difference reported in 1982-1985 and in 1995-2009. Few strains of 1980 remained close to variant CPV-2a (11 strains) and variant of 2b (2 strains),while there were no fundamental differences found in genetic profiles of the strains isolated from 1995-2009, which were classified as belonging to variant 2c.

Pinto *et al.* (2012) tested 144 stool samples from symptomatic dogs by involving PCR to target CPV-2 gene. In a result, 29.2% (42/144) found to be positive. By sequence analysis 78.6% isolates were CVP-2c, 19% as CVP-2b and 2.4% as CVP-2a. They proposed the maximum prevalence of CVP-2c in Brazil.A phylogenetic analysis revealed that there was similarity found in other countries and type 2c has become the chief type of virus strain circulating in Brazil.

Zhao *et al.* (2013) reported that, six of thirteen PCR positive isolates are phylogenetically close to the European North and South American isolates reported from 1993 and 2010.

Singh *et al.* (2014) reported phylogenetic analysis of CPV isolated from Mathura, India. Phylogenetic and molecular evolutionary examination were conducted using MEGA version 4.0. They reported that minimum divergence among themselves and also showed minimum divergence from their ancestors, such as MEV, this indicated that they recorded marginal divergence among present and ancestor strains.Study also divulged that canine parvovirus-2 variants may constitute a potential warning to canine populations.

Mukhopadhyay *et al.* (2014) studied on CPV with VP2 gene in India with 85 samples in a result based on sequences of VP2 gene,after Sequencing of these samples identified the predominant CPV strain as CPV-2a (Ser297Ala) with one CPV-2b (Ser297Ala) and another one was CPV-2a variant strain (Ser297Gly). They identified major prevalence of CPV-2a. As per phylogenetic analysis, during 2011 most of the reports are given by Tamil Nadu and Maharashtra region and in 2012 it was from Northern India. They were found to be grouped together with CPV-2a of China and India the most.

Yang *et al.* (2015) investigated two isolates (QIACP1403 and QIACP1404) and reported that they remained closely associated phylogenetically with strain CPV-BM11 retrieved from Chinese dog in 2011.

Kulkarni.(2016) sequenced nine PCR positive samples by involving Muscle multiple sequence alignment in MEGA7 software. Phylogenetic analysis done by using Bayesian time-scaled.Samples identified as CPV-2a where all grouped in CPV-2a clade and thus reported that CPV-2a already remained prevalent in the circulating part of Parbhani,Maharashtra.

Thomas *et al.* (2017) reported based on phylogenetic analysis that VP2 gene of CPV-2 of Northern India.Positive samples were subjected to oligonucleotide sequencing and these isolates were found to be similar to CPV-2a excluding at positions 264, 297 and 440 amino acid residues, found that they shared close relationship with sequences of China. These sequences remained variant in the amino acid position 264, 297 and 440 and typed as variant of CPV-2a.

Ahmed *et al.* (2018) reported that VP2 gene of CPV isolated from Dogs in Pakistan found to be sequence aligned. Phylogenetic trees were initiated by aligning the molecules using CLUSTAL-W, and by Maximum likelihood method of phylogenetic tree construction with 1000 bootstrap replications in MEGA7 program, resulted phylogenetically resembles their closeness with Chinese, Indian and Pakistani isolates especially of CPV-2a strains. This data directed towards endemic situation of the region.

2.13 Isolation of Canine *Parvovirus* using cell line:

Parthiban *et al.* (2011) by involving CRFK cell lines, PCR positive isolates were subjected to virus isolation. From total 18 samples about 3 samples reported with mild cytotoxic effect that resulted in rounding, increased granularity and detached cells observed after third passage.Rounding and degenerative changes observed after 72 hrs. of post infection in CRFK cell lines.

Puentes *et al.* (2012) used CRFK cell line for characterized canine parvovirus type 2c.Total twelve samples from two samples were subjected for isolation. Result observed in the form of rounding of cells as cytopathic effect. Presence of virus further supported by PCR and HA tests.

Zhao *et al*. (2013) tested 31 faecal samples, three samples of them found to be positive for CPV-2a. These virus load inoculation produced typical CPE in the form of rounding, detachment of cells and increased granularity and cells remained detached and second blind passages in F18 cell line. Further cytopathic effect was confirmed by electron microscopy.

Kaur *et al.* (2015) involved Nested PCR samples (n=50) for isolation of CPV in MDCK cell line. Monolayer observed after 24-48 hours. Among them, five NPCR samples are cytopathic in nature showing typical cytopathic effect. The

CPE remained featured with rounding of cells within 24hr, cell clumping with 48hr and cell detachment after 72 hours of post inoculation.

Brindalakshmi *et al.* (2016) reported the use of CRFK cell lines for the isolation of CPV. Virus get adopted in CRFK cell line and after infecting cell line with virus they were produceCPE in cell line such as Rounding and degenerative changes after third passage level in 72 hrs of post inoculums, and confirmation done by using PCR.

Vieira *et al.* (2017) obtained testing samples of *parvovirus* from dogs living in close proximity of human-wildlife interface located in Atlantic forest biome; Brazil. They observed that out of 100 samples tested, 67 (67%) samples of healthy dogs exhibited CPE on cell lines in the form of cell rounding and partial lysis of the monolayer in comparison to control.

MATERIAL AND METHODS

3.1 Material

3.1.1 Place and Facilities

The proposed research work was conducted at the Department of Veterinary Microbiology and Animal Biotechnology Teaching and Research Cell, Nagpur veterinary college, Nagpur.

3.1.2 The organism and test strain:

Megavac 7 in 1 (Indian Immunological) contain Live Canine Distemper, Adenovirus (CAV-2), Parvovirus, Para influenza and Inactivated Adenovirus (CAV-1), Leptospirosis Vaccine, available in commercial market, Nagpur, Maharashtra which was used as a pragmatic control.

3.1.3 Biological, chemicals and scientific instruments.

The molecular biology and analytical grade chemical and reagents used in the current study were acquired from Himedia, Sigma (USA), Promega (USA), Invitrogen and Qiagen. Glassware used in study were obtained from Borosil^{TM},India

3.1.4 Clinical samples:

Total 91 faecal samples/rectal swabs were collected from dog exhibiting clinical sign like, vomition, anorexia, high temperature, depression, and gastroenteritis, hemorrhagic enteritis, from various clinics located in and around Nagpur. Samples were collected along with history, age, sex, breed, vaccination status of pups was also noted during sample collection. Sample collected by using sterile swab and stored in -20°C for further processing.

Table 1: Details of Samples collection from in and around Nagpur

Sr No.	Date	Place	Sex	Age	Breed	Vaccination Status	Sample code
1	20/5/2017	Roy Pet Clinic, Nagpur	Male	2	Labrador	NV	D1
2	17/6/2017	Marwa clinic,Nagpur	Male	3	German Shepard	V	D2
3	17/6/2017	Dr. Marwa clinic,Nagpur	Male	1.5	German Shepard	V	D3
4	17/6/2017	Dr. Marwa clinic,Nagpur	Female	3	German Shepard	NV	D4
5	17/6/2017	TVCC, Nagpur	Male	3M	German Shepard	V	D5
6	20/6/2017	TVCC, Nagpur	Female	6	Non descript	NV	D6
7	20/6/2017	TVCC, Nagpur	Female	4	Pomeranian	NV	D7
8	20/6/2017	TVCC, Nagpur	Female	3	German Shepard	NV	D8
9	25/6/2017	TVCC, Nagpur	Male	6	Doberman	V	D9
10	25/6/2017	TVCC, Nagpur	Female	3	Labrador	V	D10
11	03/7/2017	TVCC, Nagpur	Male	1.5	German Shepard	V	D11
12	03/7/2017	Mahajan pet clinic	Male	2	Non descript	V	D12
13	03/7/2017	Mahajan pet clinic	Female	2.5	Non descript	NV	D13
14	03/7/2017	Mahajan pet clinic	Female	6	Labrador	V	D14
15	03/7/2017	Mahajan pet clinic	Female	2	Labrador	NV	D15
16	07/7/2017	TVCC, Nagpur	Male	3	German Shepard	V	D16
17	07/7/2017	TVCC, Nagpur	Female	3.5	German Shepard	NV	D17
18	07/7/2017	TVCC, Nagpur	Female	3	German Shepard	V	D18
19	07/7/2017	TVCC, Nagpur	Female	6	Doberman	V	D19
20	07/7/2017	Dr. Marwa clinic,Nagpur	Male	1	Doberman	NV	D20
21	07/7/2017	Dr. Marwa clinic,Nagpur	Male	9	Labrador	NV	D21
22	07/7/2017	Dr. Marwa clinic,Nagpur	Female	6	Doberman	V	D22
23	15/7/2017	Dr. Marwa clinic,Nagpur	Male	6	Pomeranian	NV	D23

24	15/7/2017	Dr. Marwa clinic,Nagpur	Male	3	German Shepard	V	D24
25	15/7/2017	Dr. Marwa clinic,Nagpur	Male	3	Doberman	V	D25
26	15/7/2017	TVCC, Nagpur	Female	6	Pomeranian	V	D26
27	03/8/2017	TVCC ,Nagpur	Male	4	Non descript	NV	D27
28	03/8/2017	Dr. Marwa clinic,Nagpur	Male	6	Non descript	NV	D28
29	03/8/2017	TVCC, Nagpur	Female	4.5	German Shepard	V	D29
30	03/8/2017	TVCC, Nagpur	Male	1.5	Labrador	NV	D30
31	08/8/2017	TVCC, Nagpur	Male	4	Labrador	V	D31
32	08/8/2017	TVCC, Nagpur	Male	3	Doberman	V	D32
33	08/8/2017	TVCC, Nagpur	Female	2	Labrador	NV	D33
34	08/8/2017	TVCC, Nagpur	Female	3.5	Non descript	NV	D34
35	13/8/2017	Dr. Marwa clinic,Nagpur	Male	3	Great Dane	V	D35
36	13/8/2017	Dr. Marwa clinic,Nagpur	Male	3	German Shepard	NV	D36
37	13/8/2017	Dr. Marwa clinic,Nagpur	Male	1	Doberman	NV	D37
38	13/8/2017	Dr. Marwa clinic,Nagpur	Male	9	Doberman	NV	D38
39	17/8/2017	Polyclinic, Nagpur	Female	3	Doberman	NV	D39
40	17/8/2017	Polyclinic, Nagpur	Female	7	Doberman	NV	D40
41	21/8/2017	Polyclinic, Nagpur	Female	1	Doberman	NV	D41
42	21/8/2017	Polyclinic, Nagpur	Female	2	Pomeranian	NV	D42
43	21/8/2017	Polyclinic, Nagpur	Male	3	Non descript	NV	D43
44	21/8/2017	TVCC, Nagpur	Female	6	German Shepard	V	D44
45	25/8/2017	TVCC, Nagpur	Female	5.5	Non descript	NV	D45
46	25/8/2017	TVCC, Nagpur	Male	3.5	Non descript	NV	D46
47	25/8/2017	TVCC, Nagpur	Male	3	Doberman	NV	D47
48	25/8/2017	Hingna road,Nagpur	Female	1.5	Labrador	NV	D48
49	25/8/2017	Hingna road,Nagpur	Male	1	Non descript	V	D49
50	25/8/2017	Hingna road,Nagpur	Female	3	Non descript	NV	D50

51	27/8/2017	Hingna road,Nagpur	Male	3	Great Dane	V	D51
52	27/8/2017	Hingna road,Nagpur	Female	1.5	Non descript	NV	D52
53	27/8/2017	Hingna road,Nagpur	Female	6	Non descript	NV	D53
54	02/9/2017	Hingnaroad,Nagpur	Female	1	German Shepard	NV	D54
55	02/9/2017	Sanjivni clinic,Nagpur	Male	2.5	Labrador	NV	D55
56	02/9/2017	Sanjivni clinic,Nagpur	Female	3	Great Dane	V	D56
57	02/9/2017	Sanjivni clinic,Nagpur	Male	4	German Shepard	V	D57
58	02/9/2017	Sanjivni clinic,Nagpur	Male	4.5	German Shepard	NV	D58
59	02/9/2017	Sanjivni clinic,Nagpur	Male	6	German Shepard	V	D59
60	12/9/2017	Raut pet clinic,Gittikhadan	Male	2	Great Dane	V	D60
61	12/9/2017	Raut pet clinic,Gittikhadan	Female	3	Non descript	NV	D61
62	12/9/2017	Raut pet clinic,Gittikhadan	Male	1.5	Doberman	NV	D62
63	12/9/2017	Raut pet clinic,Gittikhadan	Male	4.5	Labrador	NV	D63
64	12/9/2017	Sanjivni clinic, Nagpur	Male	5	German shepard	NV	D64
65	17/9/2017	Sanjivni clinic, Nagpur	Female	5	Great Dane	V	D65
66	17/9/2017	Sanjivni clinic ,Nagpur	Female	5	Labrador	NV	D66
67	17/9/2017	Raut pet clinic,Gittikhadan	Female	5.5	Pomeranian	V	D67
68	17/9/2017	Raut pet clinic,Gittikhadan	Female	2.5	Great Dane	NV	D68
69	26/9/2017	Sanjivni clinic ,Nagpur	Male	4	Pomeranian	NV	D69
70	26/9/2017	Sanjivni clinic, Nagpur	Male	4	Doberman	NV	D70
71	26/9/2017	Sanjivni clinic, Nagpur	Male	3	Non descript	NV	D71
72	26/9/2017	TVCC, Nagpur	Male	3	German Shepard	NV	D72
73	5/10/2017	TVCC, Nagpur	Male	6	Doberman	NV	D73
74	5/10/2017	TVCC, Nagpur	Female	6	Non descript	NV	D74
75	5/10/2017	TVCC ,Nagpur	Male	2	Doberman	NV	D75
76	5/10/2017	TVCC ,Nagpur	Male	4	Pomeranian	NV	D76
77	13/10/2017	Mahajan pet clinic	Female	4	German Shepard	NV	D77

78	13/10/2017	Mahajan pet clinic	Male	3.5	Non descript	NV	D78
79	13/10/2017	Mahajan pet clinic	Male	6	Great Dane	NV	D79
80	13/10/2017	Bhojne pet clinic	Male	5	Great Dane	NV	D80
81	13/10/2017	Bhojne pet clinic	Male	1	Non descript	NV	D81
82	13/10/2017	Bhojne pet clinic	Male	6	Pomeranian	NV	D82
83	6/12/2017	TVCC, Nagpur	Female	7	Non descript	NV	D83
84	18/01/2018	Marwa clinic,Nagpur	Female	1.5	German Shepard	NV	D84
85	23/01/2018	Marwa clinic,Nagpur	Male	5.5	Non descript	NV	D85
86	26/02/2018	TVCC, Nagpur	Male	2	Non descript	NV	D86
87	26/02/2018	TVCC, Nagpur	Male	4	Pomeranian	NV	D87
88	26/02/2018	TVCC, Nagpur	Male	4	German Shepard	NV	D88
89	26/02/2018	TVCC, Nagpur	Male	9	Pomeranian	NV	D89
90	26/02/2018	TVCC, Nagpur	Male	4	Non descript	NV	D90
91	03/03/2018	TVCC, Nagpur	Female	7	Non descript	NV	D91

3.1.5 <u>Clinical observations</u>

Detailed clinical examination was carried out for all the diarrheic dogs. The detailed history of the case, clinical parameters such as rectal temperature, color of conjunctival mucus membrane, respiratory rate and heart rate, type of diarrhea and the level of dehydration were recorded.

3.1.6 <u>Hiculture Collecting Device (VS)</u>

Hiculture Collecting Device (VS) was used for collection of fecal samples. The device consisted of sterile transport viscose swab with polypropylene stick with Size 150 × 12 mm diameters. (M/S Himedia Laboratories Pvt. Limited, Mumbai, India).

3.1.7 Equipments

Balance: Balance of Contech,India is used for weighing reagents in study

Vortexer (Genei,Bangloare)

Centrifuge machine (Thermo scientific,India)

Autoclave (M.C.Dalal,india)

Hot air oven (M.C.Dala,india)

Refrigerator 4^0C (LG,India)

Deep freezer (-20^0C) (Blue star,India)

ULT freezer (-70^0C),(Thermo scientific,india)

Hot Water bath (Memmert, Germany)

Micropipettes (Eppendrof, Germany and Fisherbrand, USA)

Spinvin (Genie, India)

Thermo cycler pros (Eppendrof, Germany)

Nanodrop (Eppendroff, Germany)

Laminar air flow (Microfilt, India)

UV transilluminator (Syngene G box, Uk)

Gel electrophoresis apparatus (Genei,Banglore)

Gel-documentation system (G-Box, genesys)

CO2 incubator (Thermoscientific,USA)

Inverted microscope (Zeiss, Germany)

3.1.8 Plastic ware and other consumables

Pipette tips (Genexy,India)

96 well U bottom micro titreplates(Tarson,India)

Centrifuge tubes 15 ml and 50 ml (Himedia,India)

PCR tubes (Axygen, USA)

Cell culture flask (NEST,USA)

Para film (bio basic inc., USA)

Syringes and needles (Dispovan,India)

Syringe filter with 0.22 um pore size (Pall corp.USA)

3.1.9 Canine parvovirus antigen rapid test kit

Ubioquick^{VET} canine parvovirus antigen rapid test kit is used in study for screening of samples.

3.1.10 Oligonucleotide primers:

The Oligonucleotide primers used for PCR to amplify VP2 gene of CPV-2 virus are given in Table-2

Table - 2. Details of Primers specific for VP2 gene of CPV used in PCR

Primer name	Sequence primers (5'-3')	Product size	Reference
CPV-2ab-F	GAAGAGTGGTTGTAAATAATT	681 bp	Sheikh *et al,(*2017)
CPV-2ab-R	CCTATATAACCAAAGTTAGTAC		

3.1.11 MDCK cell line

MDCK cell lines were procured from National Centre For Cell Sciences (NCCS), Pune, Maharashtra for present study.

3.2 Methodology

3.2.1 Haemagglutination (HA) test

3.2.1.2 1% Porcine Erythrocytes

The blood samples were collected from non-descript, local breed of adult swine in Alsever's solution in equal quantity of both solution and blood (1:1,v/v)and kept at 4^0C for 48 hrs. The centrifugation was carried out at 1500 rpm for 20 min and washed the RBCs thrice in Phosphate buffered saline (PBS) having pH 7.2, and the porcine erythrocytes were stored at 4^0C for further use.

3.2.1.3 Preparation of samples

The fecal samples collected from suspected cases were immersed in PBS. The samples were then centrifuged at 2500 rpm for 20 min at 4^0C. The

supernatant were separated and preserved at -20^0C and these supernatant used for further HA test.

3.2.1.4 Procedure

The Haemagglutination test (HA) test performed as per OIE .

- Take 25 microliter of PBS into each well of U bottom microtitre plate.
- Add 25 microliter of virus suspension in the first well.mix the contents properly and transfer 25 microlitres to the next well. Again mix the suspension and transfer to next well, thus carry out two fold dilution of virus up to ninth well.
- Discard 25 microliter suspension from the ninth well.
- The eleventh well is maintained as RBC control and 12th well as antigen control.
- Add 25 microliter of 1% porcine RBC suspension in all the wells in a row.
- Shake the plate well and keep it at 4^0C for 30 minutes.
- Read the test in 30, 45 and 60 minutes of interval and record the result.

Interpretation of the result:

- HA titer of 64 and above was considered as positive for CPV antigen.

3.2.2 Detection of Canine Parvovirus Ag

- Fecal samples were screened for CPV antigen ubioquickVET Canine Parvovirus antigen rapid test kit as per Folitse *et al.*(2017)

3.2.2.1 Procedure:

- The Rectal swab inserts into assay diluents and agitates it sufficiently in order to good sample extraction; make sure that suspension does not contain any precipitation by centrifuging it if required.
- Placed it for few minutes.
- Add 3 drop of the specimen into sample hole “s”.
- Wait for 10 minutes and interpret the result.
- The result is considered invalid after 15 minutes.

3.2.2.2 <u>Interpretation of the result:</u>

- The sample was considered positive for the presence of CPV if two red bands appear one in the control line (C) and the other in the test line (T).
- The sample was considered negative for CPV if one red band appears in the control line(C) with no apparent band in the test line (T).
- If no purple band appears in the control line(C) or if a band appears in the test line (T) but not in the control line(C), then the test is invalid.

3.2.3 Nucleic Acid Detection Methods

3.2.3.1 <u>Extraction of DNA from Vaccine.</u>

Megavac 7 in 1 (Indian Immunological) available in commercial market, Nagpur, Maharashtra, was used as a pragmatic control. The DNA was extracted from these vaccines using DNA extraction kit. (Promega kit,USA)

3.2.3.2 <u>Extraction of DNA from the faecal samples</u>

The DNA was extracted from faecal samples by using DNA extraction kit. (Promega kit,USA)

3.2.3.3 <u>Processing of faecal samples</u>

The fecal sample were collected in phosphate buffer saline (pH=7.2) to make suspension followed by centrifugation at 2000 rpm for 10 minutes, to remove shaggy particles and cellular debris. The clarified suspension was stored at -20^{0}C till further use.

3.2.3.4 <u>Procedure for extraction of viral DNA from Vaccine as per kit protocol</u>

- Three hundred microlitre of vaccine was taken in tube.
- Add two hundred microlitre of proteinase K solution and vortex it properly.
- Add four hundred microlitre of cell lysis buffer, vortex for 10 sec.
- Incubate it at 56^{0} C for 30 minutes.
- After that add five hundred microlitre of binding buffer (BBA), vortex for 10 sec.

- Place a Relia prep ™ binding column into a collection tube for each sample.
- After that add liquid portion of sample and to the binding column and centrifuge for 1 minute at maximum speed (rpm).
- Discard the swab.
- Place the column into a fresh collection tube.
- After add five hundred microlitre of column wash solution given in kit to the column and centrifuge at maximum speed for 2 minutes. If the sample is still visible on top of the membrane, centrifuge the column for another minute.
- After that place the column in a fresh collection tube.
- Repeat the washing step twice for a total of 3 washes.
- Place the column into a clean ,labeled 1.5 ml microcentrifuge tube and add fifty microlitre of nuclease free water and centrifuge the column for 1 minute at maximum speed.
- Then discard the column and the genomic DNA of vaccine can be placed at -20^{0}C.

3.2.3.5 <u>Procedure for extraction of viral DNA from rectal swab.</u>

- Take a rectal swab in PBS solution, vortex it properly.
- Centrifuge the tube at 2000 rpm for 15 minutes.
- Collect the supernatant in another vial.
- Add two hundred microlitre of proteinase K solution and vortex it properly.
- Add four hundred microlitre of cell lysis buffer, vortex for 10 sec.
- Incubate it at 56^0 C for 30 minutes.
- After that add five hundred microlitre of binding buffer (BBA), vortex for 10 sec.
- Place a Relia prep ™ binding column into a collection tube for each sample.

- After that add liquid portion of sample in to the binding column and centrifuge for 1 minute at maximum speed (rpm).
- Discard the swab.
- Place the column into a fresh collection tube.
- After add five hundred microlitre of column wash solution given in kit to the column and centrifuge at maximum speed for 2 minutes. If the sample is still visible on top of the memebrane, centrifuge the column for another minute.
- After that place the column in a fresh collection tube.
- Repeat the washing step twice for a total of 3 washes.
- Place the column into a clean, labeled 1.5 ml microcentrifuge tube and add fifty microlitre of nuclease free water and centrifuge the column for 1 minute at maximum speed.
- Then discard the column and the genomic DNA of vaccine can be placed at -20^{0}C.

3.2.3.6 Standardization of Polymerase Chain Reaction (PCR) for amplification of VP2 gene

The extracted viral DNA was amplified by PCR assay using VP2 gene specific primers. The PCR assay for VP2 gene was standardized using gradient PCR to obtain the maximum amplification of partial length of 681 bp product of the gene. The reaction was set up as follows.

Components	Volume/ reaction
Template DNA	2 µl
5X PCR buffer	10 µl
Mg Cl_2 (50 mM)	3 µl
dNTPs (10 mM each)	1 µl
Taqpolymeras(5 units/µl)	1 µl
Forward Primer	2 µl
Reverse Primer	2 µl
Nuclease free water	29 µl
Total	50 µl

DNA from a vaccine was used as a positive control and fecal sample from healthy dog kept as a negative control. All these ingredients are mix properly by vortexing.

The reaction was carried out using following cycle conditions for VP2 gene.

Steps	Temperature	Time
Initial Denaturation	94^0C	5 Min
3-step cycling for 30 cycles		
Denaturation	94^0C	1 Min
Annealing	50^0C	2 Min
Extension	72^0C	2 Min
Final extension	72^0C	10 Min
cooling	4^0C	-

The PCR product were removed from the Thermal cycler ProS (Eppendroff, Germany) and analyzed by gel electrophoresis.

3.2.3.7 <u>Confirmation of VP2 gene amplicons by agarose gel electrophoresis</u>

Gel casting plates were assembled and kept on leveled surface and open sides were then sealed with adhesive tape. Gel comb was set inside the chamber having 1 mm base above the platform. Boiled 1% agarose (Sigma Aldrich) which was prepared in 0.5X Tris borate electrophoresis (TBE) buffer and then added with ethidium bromide when temperature reaches to about 50^oC with final concentration at 0.5 µg/ml. Molten agar then poured on casting platform and allowed to solidify. After solidification carefully gel comb was removed along with tape. The plate was then submerged in the electrophoresis tank with well at the cathode end of the tank with sufficient quantity (about 1 mm level) of electrophoresis buffer (TBE, 0.5X) above the surface of the gel.

About 5 µl of PCR product was then mixed with 3 µl of bromophenol blue (6X) (Sigma aldrich) gel loading dye and loaded into well. In another well, 100 bp DNA ladder (Sigma aldrich) was loaded as a marker. Further electrophoresis was carried out at 5 volts/cm and at end of migration gels were monitored under UV light for banding pattern by using UV transilluminator (Syngene G box, Uk).

3.2.4. Sequencing

One sample randomly selected On the basis of amplicon of PCR, The PCR product were selected and were commercial sequenced from Triyad Genomic, Coimbatore,Tamilnadu India and submitted to DDBJ.

3.2.4.1. Sequence and Phylogeny analysis

The obtained sequences from the field sample were subjected to BLAST analysis with GenBank database sequences using BLASTn algorithm available at NCBI blast (http://blast.ncbi.nlm.nih.gov/Blast) the Clustal-W (CLUSTAL 2.1 multiple sequence alignment) as per the method of Kaur*et al.,*(2015) to confirm the presence of the gene specific to CPV. The nucleotide sequences of VP2 gene fragment of CPV were aligned using default parameters of muscle alignment implemented in MEGA 6.0 software (http://www.megasoftware. net/) as per the method of Tamura *et al.,* (2013) with 32 sequences including sequences of Indian and foreign CPV isolates retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/index.html).

Molecular Phylogenetic analysis was carried using Maximum Likelihood method based on the Kimura 2 –parameter model (Kimura., 1980).Initial tree (s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (0.1160) the tree is drawn to scale, with branch lengths measured in number of substitutions per site .All positions containing gaps and missing data were eliminated.

3.2.5 Isolation of Canine parvovirus-2

Virus Isolation is done by using Madin Darby Canine Kidney (MDCK) Cell Line which was procured from National Centre of Cell Science, Pune (India).The fecal sample positive for canine parvovirus by PCR used for virus isolation in MDCK cell line.

3.2.5.1Cell line and culture

MDCK cell line was procured from National Centre of Cell Science, Pune (India) and was maintained in the Department of Veterinary Microbiology and

Animal Biotechnology T & R Cell, Nagpur Veterinary Collage, Nagpur. The cell culture flask having MDCK cell line had been transported in maintenance medium as monolayer in cell culture flask. The cell culture flask (25 cm^2 cell culture flask) was kept in incubator at 37ºC with 5% CO_2. Cells were observed under inverted microscope.

3.2.5.2 <u>Subculturing of MDCK cells</u>

Observed the cell under inverted microscope for confluent monolayer of MDCK cell line in cell culture flask was subjected to subculturing. The GM DMEM in the cell culture flask was discarded and monolayer of MDCK cells was given two washings with PBS (pH 7.2). Then, 1 ml of TPGV solution was added to cover the surface of cells and the flask was kept at 37ºC for 30 seconds for the detachment of the cells. Again, 2 ml of TPGV was put in to flask and kept for 1 min, then medium was removed and a film of TPGV was left in the flask. Flask was kept at incubator at 37ºC with 5% Co_2 for 5 min. Detachment of cell monolayer could be visualized against the tube light. Once the cell detachment was observed under inverted microscope, GM DMEM was added to neutralise the effect of trypsin. Vigorous pipetting was done for effective detachment and new flasks were seeded with these cells at the split ratio of 1:2. The new flasks were supplemented with 5 ml of GM DMEM each and incubated at 37ºC for obtaining a complete monolayer.

3.2.5.3 <u>Preparation of virus inoculums/ sample processing</u>

The collected fecal sample were suspended in 10% (W/V) phosphate buffered saline (PBS, pH 7.2),was centrifuged at 10000 rpm for 10 minutes rpm at 4°C to collect the supernatant. Collected supernatant then filter through 0.45 membrane syringe filter, and these filtrated mixed with equal volume of DMEM medium contain 2% FBS and 10 ug/ml crystalline trypsin.After proper mixing incubate it at 37° C for 60 min.after incubation 1 ml of mixture inoculated into cell culture flask with 80-90% confluent monolayer of MDCK.Kept it for 1 hour of incubation in CO_2 incubator at 37^0C with 5% CO_2 for complete adsorption. Washed the cells 3 times with plain DMEM .After washing over layered the cells with maintenance medium contain 1 ug/ml crystalline trypsin and placed the flask in CO_2 incubator at 37^0C with 5% $CO_{2.}$and examined under inverted microscope daily up to 5 days for the appearance of cytopathic effects (CPE). One un-

infected monolayer cell culture flask was kept as normal cell control by adding maintenance media for maintenance of the MDCK cells.

3.2.5.4 Harvesting of the virus

After incubation for 5 days for the CPE, the cell line irrespective of whether CPE appeared or not was subjected to two cycles of alternative freezing and thawing. The samples which did not exhibit CPE in first passage were further subjected to 2nd and 3rd passage. The cell culture fluid was collected in micro centrifuge tube and stored at -20°C to be used for further passaging of the samples.

3.2.5.5 PCR of cell culture passage samples

The cell culture fluid of the sample which showing CPE obtained were screened for the confirmation of canine parvovirus by PCR. The cell culture fluids were subjected for DNA extraction using DNA extraction kit (Promega, USA). As described earlier 3.2.3.5.The PCR of the sample was done using same cycling condition and confirmation of VP2 gene amplicon by agarose gel electrophoresis was done as described earlier 3.2.3.6 and 3.2.3.7.

RESULT AND DISCUSSION

Canine parvovirus-2 is one of the most infective pathogenic viruses, highly contagious and deadly disease of dog specially pups, and it can prove fatal if untreated. It is responsible for causing diarrhea accompanied by severe hemorrhagic enteritis and myocarditis in dogs. In 1978, Canine parvovirus emerged as one of the serious viral infection in dog population causing heavy mortality.

Canine parvovirus cause blood tinge dirrahoea, vomittion, dehydration and infected dog serve as a potential source of infection to others.Immunosuppression is one of the major causes of CPV, which may lead to reduce immunity of dog against other infectious diseases.(Kaur *et al.*, 2015).

In India, CPV-2 was first recorded in Chennai city by Balu and Thangraj in 1981.Chennai has large number of populations of canines, hence there is high risk of infection, several occurrences of diseases have been reported from various states of India from both vaccinated as well as unvaccinated dogs.

In the 1980s, the original CPV-2 underwent antigenic changes and was totally replaced by novel antigenic variants nominated as CPV-2a, CPV-2b and more recently CPV-2c and the original CPV-2 virus is tough to record for its presence in canine populations and existed only in vaccine formulations. It is still being formulated in the preparation of commercial vaccines. Therefore, effective vaccine treatment against CPV infection in dogs remains ineffective to protect pups against new CPV antigenic variants. Thus, vaccine used currently to prevent CPV-2 infection in dogs fails to effectively protect pups against the new CPV antigenic variants.

Although the clinical cases of CPV are diagnosed by clinical signs, the confirmation must be done at laboratory. In a testing setup, number of regular methodologies such as hemagglutination inhibition (HI), ELISA, PCR, virus isolation, electron microscopy, immunochromatography, Immunofluorescent test (IFT) and agarose gel precipitation test are very common. However, a prompt diagnosis from the laboratory is beneficial to the clinical practitioner for immediate initiation of suitable therapy.

There are various diagnosis methods including both conventional and molecular, seeing the severity of infection, present study was undertaken by using various diagnostic methods include Haemagglutination test (HA), antigen detection kit, Polymerase chain reaction, genome sequencing with phylogenetic analysis along with isolation of CPV in cell line.

4.1 Haemagglutination test

Haemagglutination test involves the haemagglutination activity of canine parvovirus, typically with the procine RBS's maintained at 4°C (Carmichael *et al.,* 1980) and by which we can differentiate CPV from *Feline panleukopenia virus* (FPV), minute virus of canines and Mink Enteritis Virus (MEV). The property of binding to the specific sialic acid receptor is important for diagnosis. HA test is rapid, specific and inexpensive results are generated in 2-4 hours. The only limitation is the need of regular supply of the reactive RBCs and check for specificity of low tittered reaction with haemagglutination inhibition assay (Senda., 1986). In the present study also, canine parvovirus took 1-2 hours to agglutinate the pig RBC at 4°C. These findings were correlating with the observations made by (Marulappa *et al.,*2009).

In the present study, a total of 91 faecal samples were collected from dog with different places of Nagpur having symptoms like diarrhoea, (Plate 1 and 2) gastroenteritis with pyrexia, vomition (Plate 3) were screened for presence of CPV-2,by haemagglutination test using porcine erythrocyte as per OIE. Samples showing mat formation considered as positive and HA titer above 32 considered as positive for HA. (Muthuraj *et al*., 2016).

4.1.1. <u>Frequencies wise prevalence of CPV infection by HA test</u>

A total 91 faecal samples were screened by HA test of them 22 (24.17%) samples agglutinated pig RBC with titer ranging from 64-1024. Nine samples shows 64 HA titer, Six samples shows HA titer 128, four sample shows 256 HA titer, Two sample shows HA titer 512 and only one sample shows HA titer 1024 (Table 3, Plate 4 and 5). The HA titer of processed faecal samples above 64 were considered as positive for the canine parvovirus (Shashidhara and Kapil.,2009). Our result was found similar with Kulkarni (2016) who processed 25 fecal sample out of these 9 sample were positive for HA showed titer >64 considered positive whereas Reddy et al. (2012) found that total 217 faecal

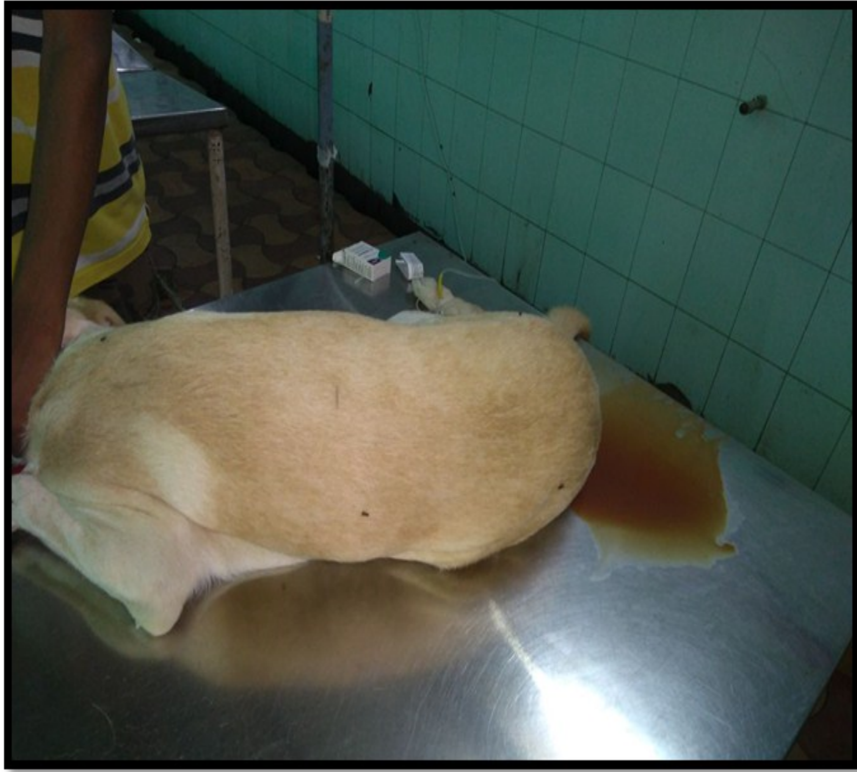

Plate 1: Dog showing Bloody diarrhea (CPV-2)

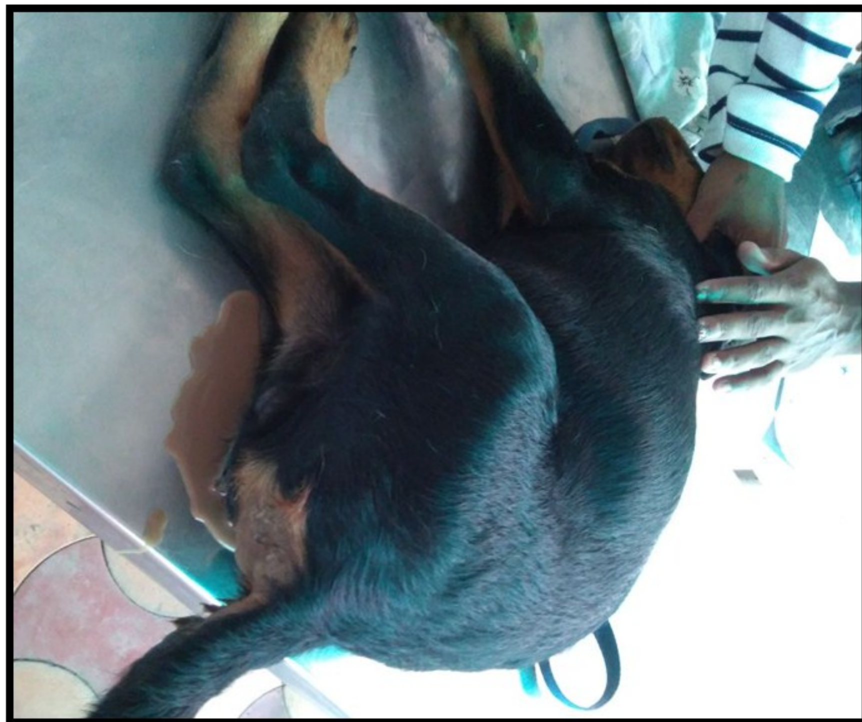

Plate 2 : Dog showing acute Bloody diarrhea (CPV-2)

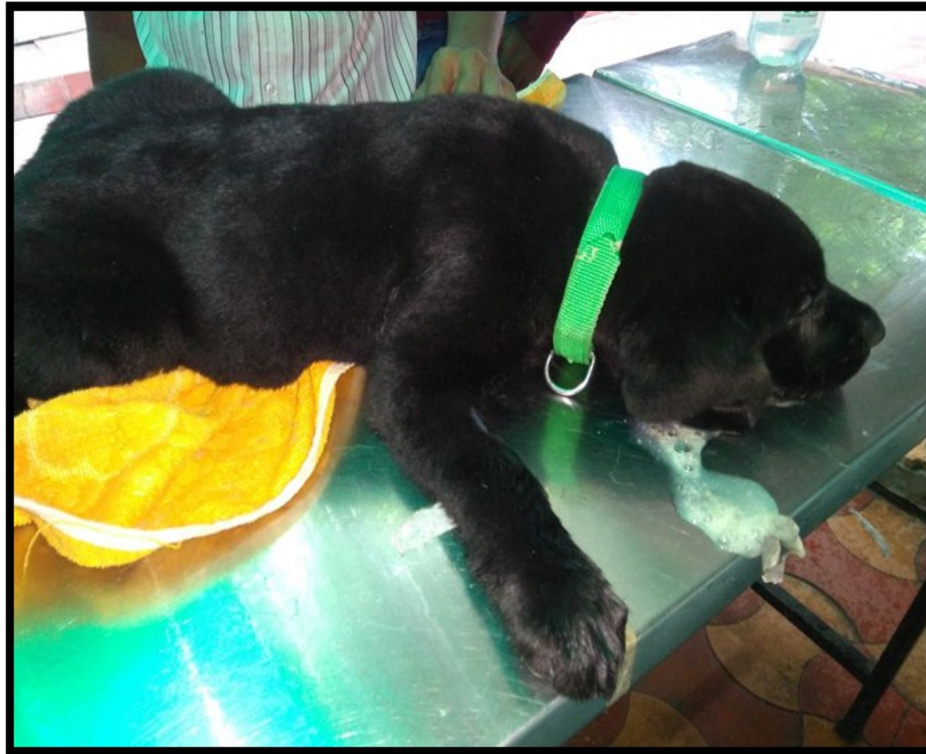

Plate 3: Dog showing Vomition (CPV-2)

samples for CPV antigen by HA test of them 72 samples were positive with titers ranging from 32-1024. Silva *et al*. (2013) were collected total 112 fecal samples out of these 32 samples tested highly positive with titers >128, eight tested positive with titers 32 and 64.Umar *et al*. (2015) were collected 198 faecal samples from dogs out of them 45 samples were found positive by haemagglutination with prevalence of 22.7 %.

Table 3: Distribution of HA titres of CPV positive faecal samples with their frequencies.

HA titre	Frequencies
64	9
128	6
256	4
512	2
1024	1
Total	22

Although epidemiological analysis is not the focus of present study, it is essential to document some observations related to age of pups, sex, breed and their vaccination status. Samples which show positive titers were distributed according to age, sex, breed and vaccination status of dogs by HA inTable 4.

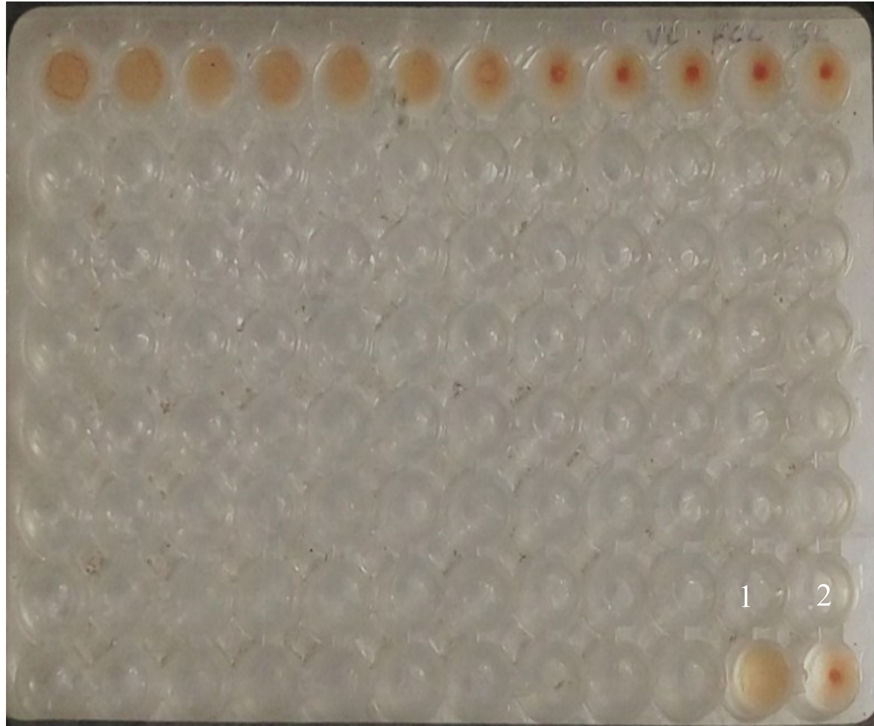

Plate 4 : HaemagglutinationTest of CPV vaccine and control with Pig RBCs.
Positive control:-1
Negative control:-2

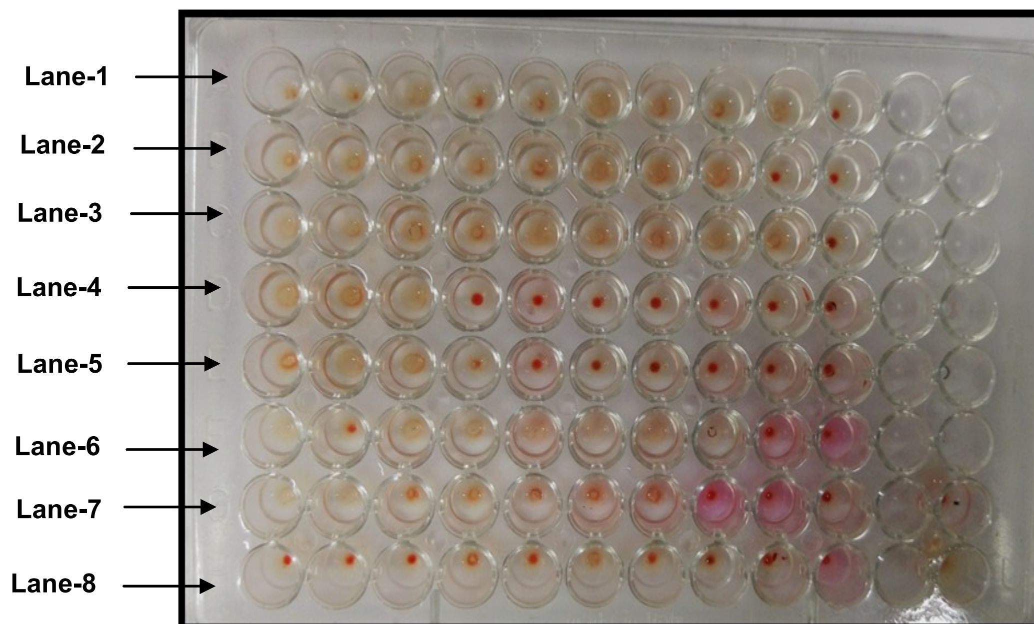

Plate 5 : Haemagglutination Test of CPV with Pig RBCs to sample
Lane 1,2,3,6 and 7 show matt formation :- positive samples.
Lane 4,5 and 8 show button formation :- Negative samples.

Table 4:Details of Age, Sex, Breed and Vaccination Status of samples positive by HA

Sr.No	Lab code	HA positive	Age	Sex	Breed	Vaccination status
1	CPV-36	+	3	M	German Shepherd	NV
2	CPV-37	+	1	M	Doberman	NV
3	CPV-68	+	2.5	F	Great dane	NV
4	CPV-32	+	3	M	Doberman	NV
5	CPV-55	+	2.5	M	Labrador	NV
6	CPV-78	+	3.5	M	Non descript	NV
7	CPV-60	+	2	M	Great dane	NV
8	CPV-12	+	2	M	Non descript	V
9	CPV-81	+	1	M	Non descript	NV
10	CPV-72	+	3	M	German Shepherd	NV
11	CPV-62	+	1.5	M	Doberman	NV
12	CPV-84	+	1.5	F	German Shepherd	NV
13	CPV-8	+	3	F	German Shepherd	NV
14	CPV-59	+	6	M	German Shepherd	NV
15	CPV-26	+	6	F	Pomerian	V
16	CPV-58	+	4.5	M	German Shepherd	NV
17	CPV-67	+	5.5	F	Pomerian	NV
18	CPV-85	+	5.5	M	Non descript	NV
19	CPV-17	+	3.5	F	German Shepherd	NV
20	CPV-23	+	6	M	Pomerian	NV
21	CPV-5	+	3	M	German Shepherd	V
22	CPV-21	+	9	M	Labrador	NV

4.1.2. Age, sex, breed and vaccination status wise prevalence of CPV-by HA.

In the present study, total 22 HA positive faecal samples were distributed according to age, sex, breed and vaccination status. Age wise distribution showed that maximum positive samples belong to age group of 0-3 (26.60%) followed by 4-6 (23.07%) and then 6-9 (14.28%).(Fig.1) highest prevalence was observed in male dog (30.18%)(Fig.2).Breed wise analysis of data indicated that highest prevalence in German shepherd (36.36%), followed by Pomerian (30 %), Great Dane (25%), Doberman (18.75%), Labrador (16.66%) and Non descript (17.39%) (Fig.3).Three samples were positive with prevalence of (17.8%) in vaccinated and (30.15%) in non vaccinated dogs. (Fig.4) .The results in respect of age were corroborated with the reports of Banja *et al.* (2002b). Younger pups showed more prevalence as they lack effective innate immunity against virus and CPV showcased close affinity for dividing cells available in the intestine of the young animals.

The present study of sex-wise prevalence is in contrast with Banja *et al.* (2002) who confirmed that sex does not influence the incidences of the CPV infection. Whereas Reddy *et al.* (2012) and Kaur *et al.* (2015) reported that male (37.30% and 63.63%) was more prone to CPV than female (27.47 and 36.36%).As per breed wise recording, current study showcased harmony with Sagar. (2005) report where he stated that Indian canine parvo virus enteritis has got the status of new emergence and few breeds like German shephard ,Labrador, Spitz and at under sever risk of parvo viral enteritis as compared to Mongrels which remain less susceptible. Similarly,Sanjukta *et al.*(2011) recorded the similar findings with 50% presence of CPV in Doberman followed by German shephard (41.1%) and Spitz (32.5%). Regarding result of vaccination status of the dogs, Deepa and Saseendranath. (2002) also reported vaccination failures in dogs due to vaccinated with killed or live virus vaccines. Possibility of ineffective vaccine response may be arising due to inadequate repetition of the booster doses of killed vaccine Hoskins. (2006), or it may be because of new genetic composition of the emerging CPV strains (Decaro *et al.,*2008).

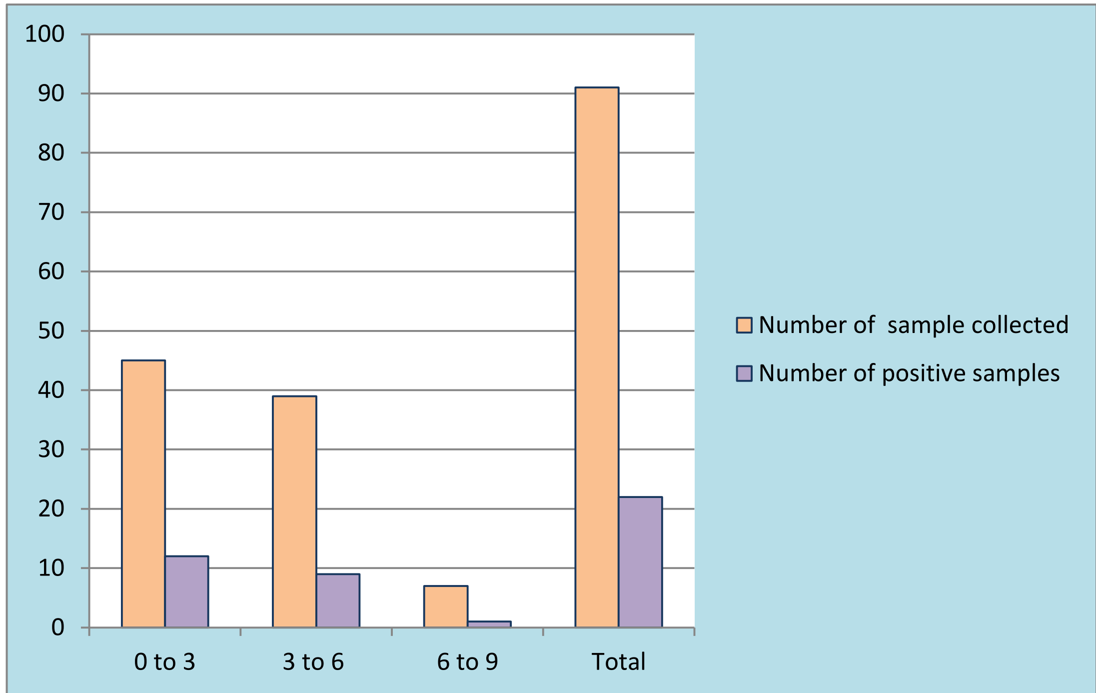

Fig. 1: **Age-wise status of Canine Parvovirus by HA**

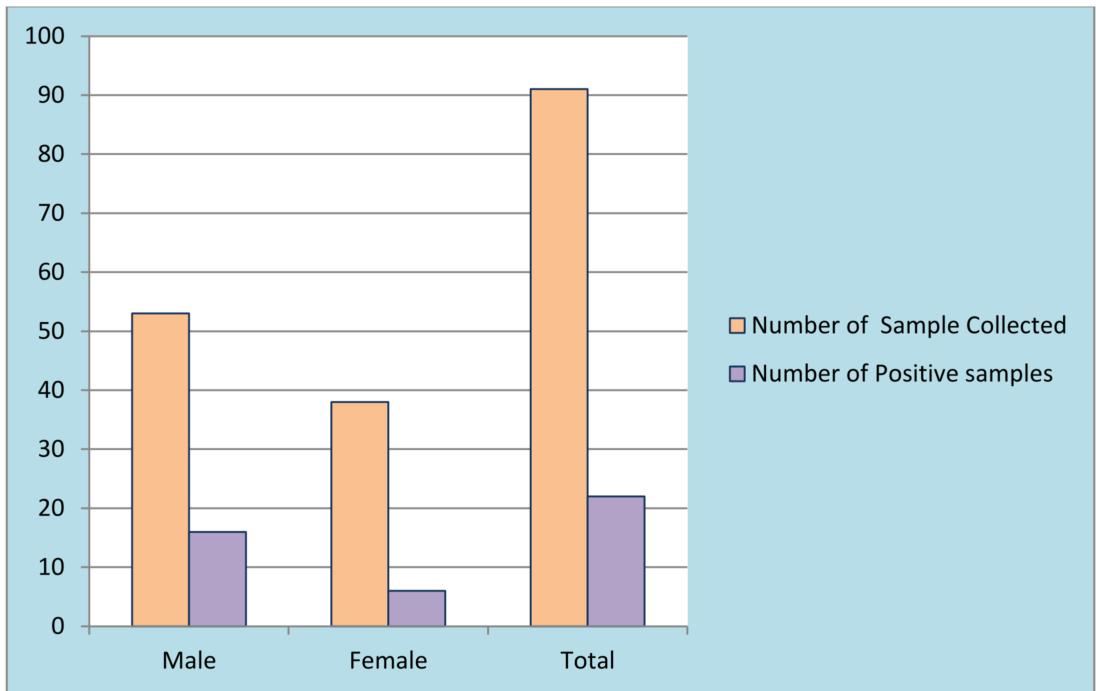

Fig. 2 : **Sex-Wise distribution of Canine Parvovirus by HA**

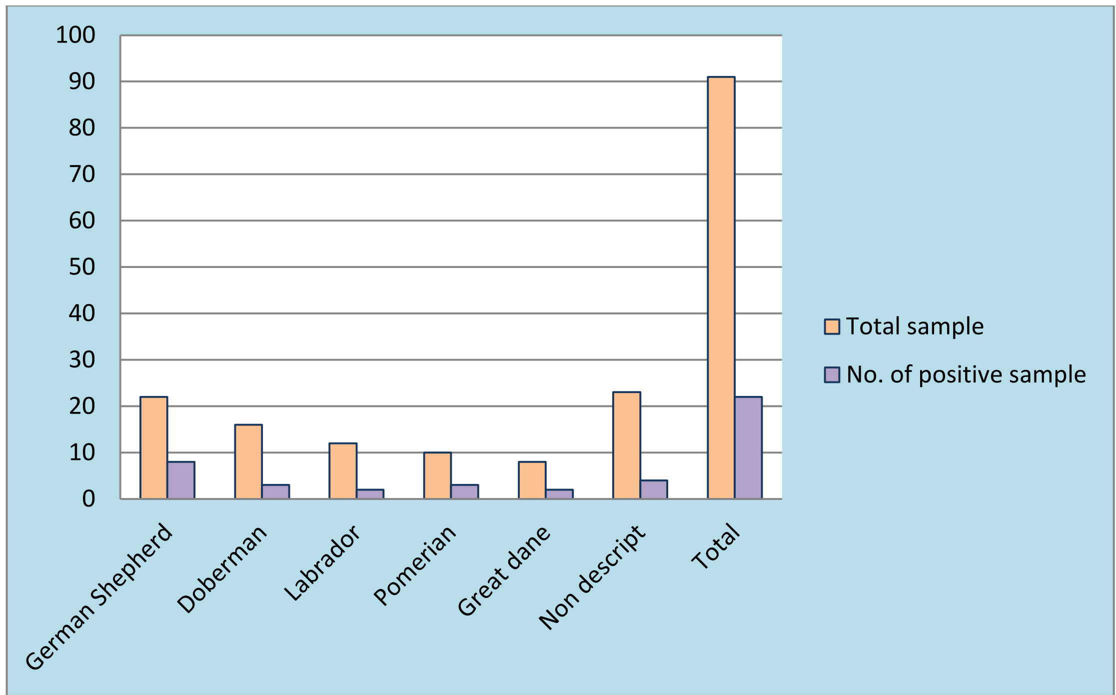

Fig. 3: Breed-Wise Prevalence of Canine Parvovirus by HA

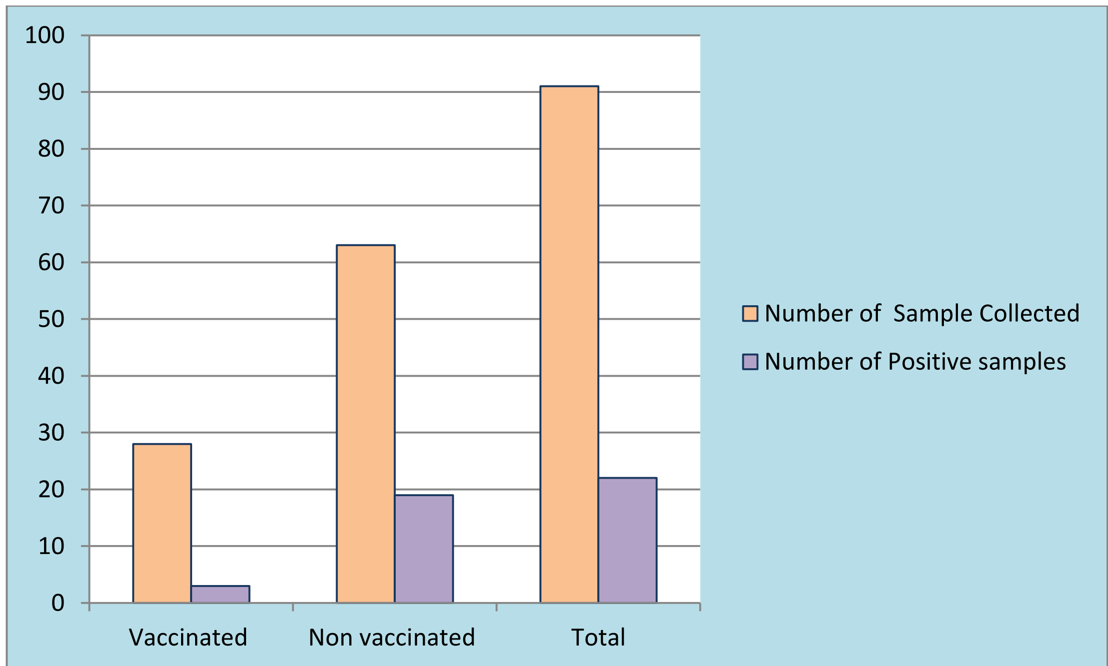

Fig. 4: Prevalence of Canine Parvovirus by HA based on the vaccination status.

4.2. Detection of CPV-2 Antigen by Kit

A rapid detection of CPV-2 infection can control secondary infection of susceptible contact animals especially in kennels and shelters and clinical diagnosis are error prone, by involving laboratory methods. CPV-2 detection could be speed up in infected dogs. Among many available tests, immunochromatography assay (ICA) rated top in the rapid detection of CPV-2 cases and also offers their high sensitivity at low cost and can be performed by any veterinarians as well as by owners (Esfandiari and Klingeborn.,2000). However, it remains affected by the viral load available in the sample and remained noneffective at low viral load.

In the present study, a total of 91 faecal samples were screened for presence CPV antigen by ubioquickVET parvovirus antigen detection rapid test, of them 34 (35.86%) samples were found positive. (Plate 6 and 7). The present findings agree with findings of Sundaran et *al*. (2015) who stated rapid immunochromatographic strip test certainly capable of detecting canine parvovirus infection.

There are diverse ways for detection of the virus antigen in canine feces by rapid antigen test kit. This enables the specific parvovirus antibodies to identify antigen in canine feces with high degree of accuracy.These results agreed with other investigators Esfandiari and Klingeborn.(2000), also same results recorded by Mosallanejad *et al.*(2008), when used the same test kit which showed high sensitivity and specificity to detect the subtypes in Iran, also Oh *et al. (*2006) used kit to detect antibodies rather than antigen.

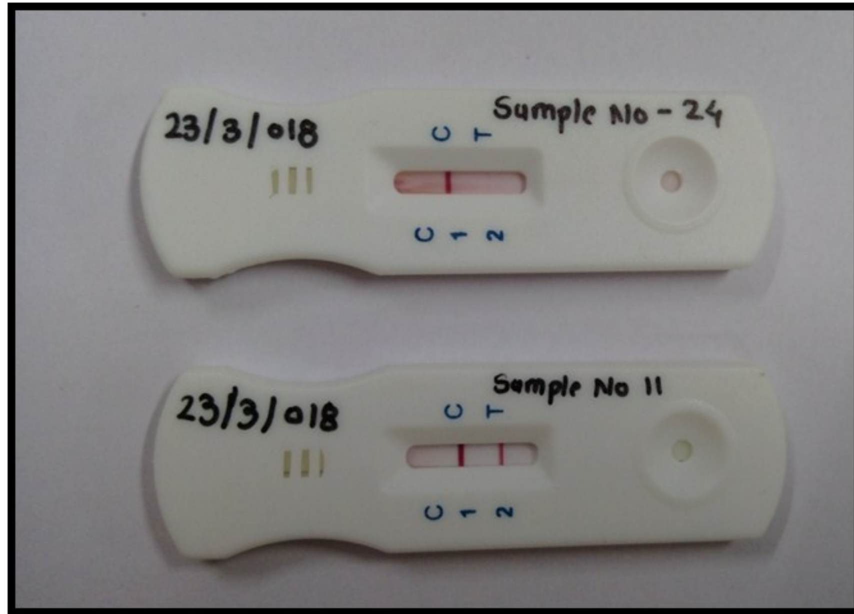

Plate 6 : Ubio quickVET Canine parvovirus antigen rapid test kit showing positive and negative reaction for CPV antigen.
Both "C" and "T" line show –Positive. (Sample-11)
Only "C" line show -Negative. (Sample-24)

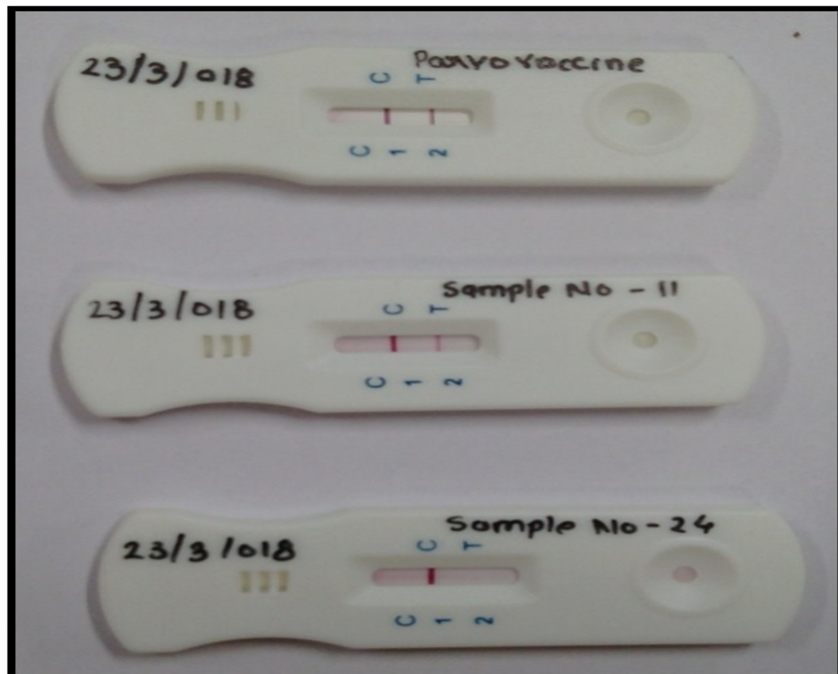

Plate 7: Sample compare with commercially available Parvovirus vaccine.
Parvovirus vaccine:-Positive
Sample No 11:-positive
Sample No 24:-Negative

Table 5:Details of Age, Sex, Breed and Vaccination Status of samples positive by Antigen detection Kit

Sr.No	Lab code	Antigen Kit	Age	Sex	Breed	Vaccination status
1	CPV-36	+	3	M	German Shepherd	NV
2	CPV-37	+	1	M	Doberman	NV
3	CPV-20	+	1	M	Doberman	NV
4	CPV-21	+	9	M	Labrador	NV
5	CPV-42	+	2	F	Pomerian	NV
6	CPV-25	+	3	M	Doberman	NV
7	CPV-47	+	3	M	Doberman	NV
8	CPV-8	+	3	F	German Shepherd	NV
9	CPV-60	+	2	M	Great Dane	NV
10	CPV-78	+	3.5	M	Non descript	NV
11	CPV-7	+	4	F	Pomerian	NV
12	CPV-18	+	3	F	German Shepherd	V
13	CPV-12	+	2	M	Non descript	V
14	CPV-43	+	3	M	Non descript	NV
15	CPV-46	+	3.5	M	Non descript	NV
16	CPV-67	+	5.5	F	Pomerian	NV
17	CPV-58	+	4.5	M	German Shepherd	NV
18	CPV-54	+	1	F	German Shepherd	NV
19	CPV-11	+	1.5	M	German Shepherd	NV
20	CPV-30	+	1.5	M	Labrador	NV

21	CPV-72	+	3	M	German Shepherd	NV
22	CPV-81	+	1	M	Non descript	NV
23	CPV-85	+	5.5	M	Non descript	NV
24	CPV-3	+	1.5	M	German Shepherd	V
25	CPV-68	+	2.5	F	Great Dane	NV
26	CPV-54	+	1	F	German Shepherd	NV
27	CPV-23	+	6	M	Pomerian	NV
28	CPV-84	+	1.5	F	German Shepherd	NV
29	CPV-41	+	1	F	Doberman	NV
30	CPV-5	+	3	M	German Shepherd	NV
31	CPV-17	+	3.5	F	German Shepherd	NV
32	CPV-12	+	2	M	Non descript	V
33	CPV-64	+	5	M	German Shepherd	NV
34	CPV-88	+	4	M	German Shepherd	NV

4.2.1. Age, sex, breed vaccination status wise prevalence of CPV-by Antigen detection kit.

In the present study, total 34 samples positive by antigen detection kit distributed according to age, sex, breed and vaccination status (Table 5). Age wise distribution showed that maximum positive samples belong to age group of 0-3 (51.11%,) followed by 4-6 (48.71%) and then 6-9 (14.28%). (Fig.5) Highest prevalence was observed in male dog (60.37%). (Fig.6) Breed wise analysis of data indicated that highest prevalence in German shepherd (72.72%), followed by Pomerian (40 %), Doberman (25%). (Fig.7) 4 samples were positive with prevalence of (14.28%) in vaccinated and (46.03%) in non-vaccinated dogs.

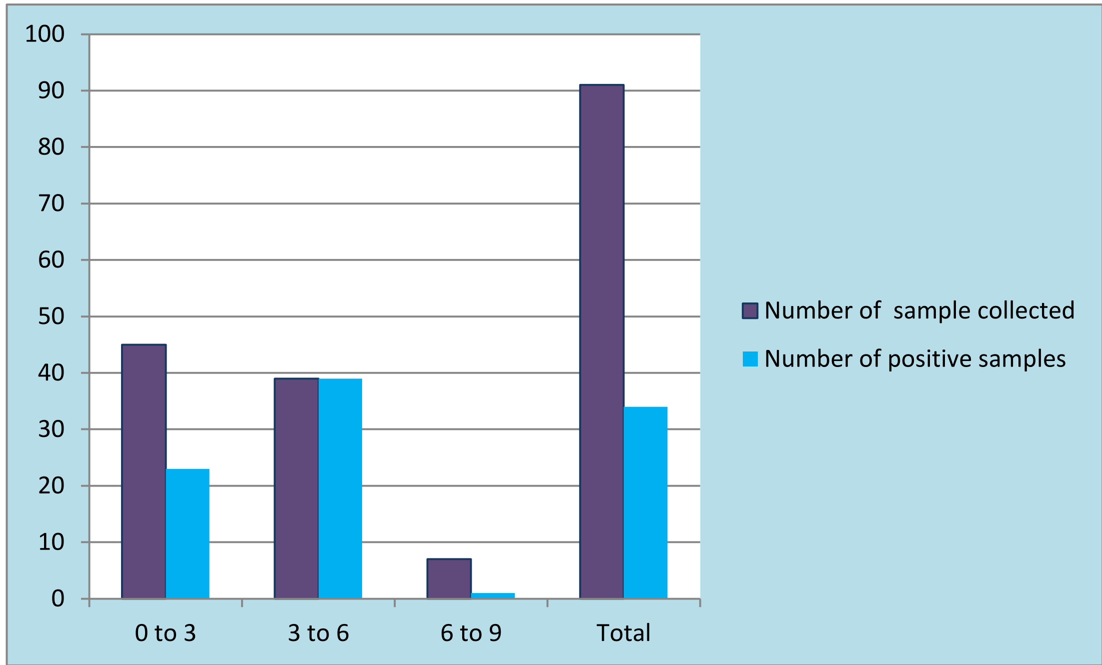

Fig. 5: Age-wise status of Canine Parvovirus by Antigen detection kit

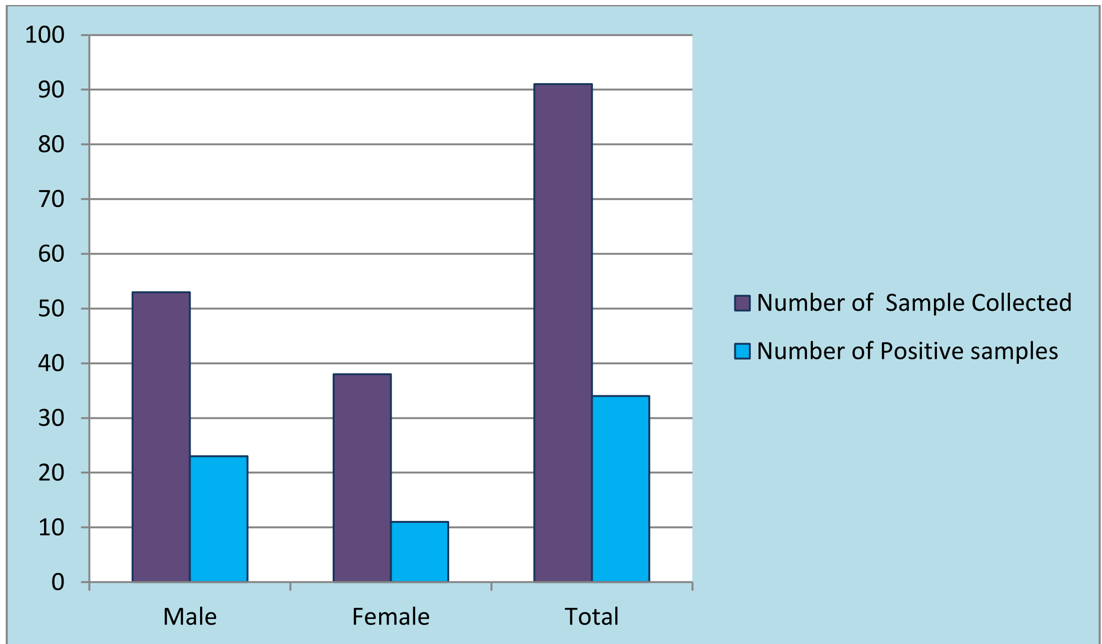

Fig. 6: Sex-Wise distribution of Canine Parvovirus by Antigen detection Kit

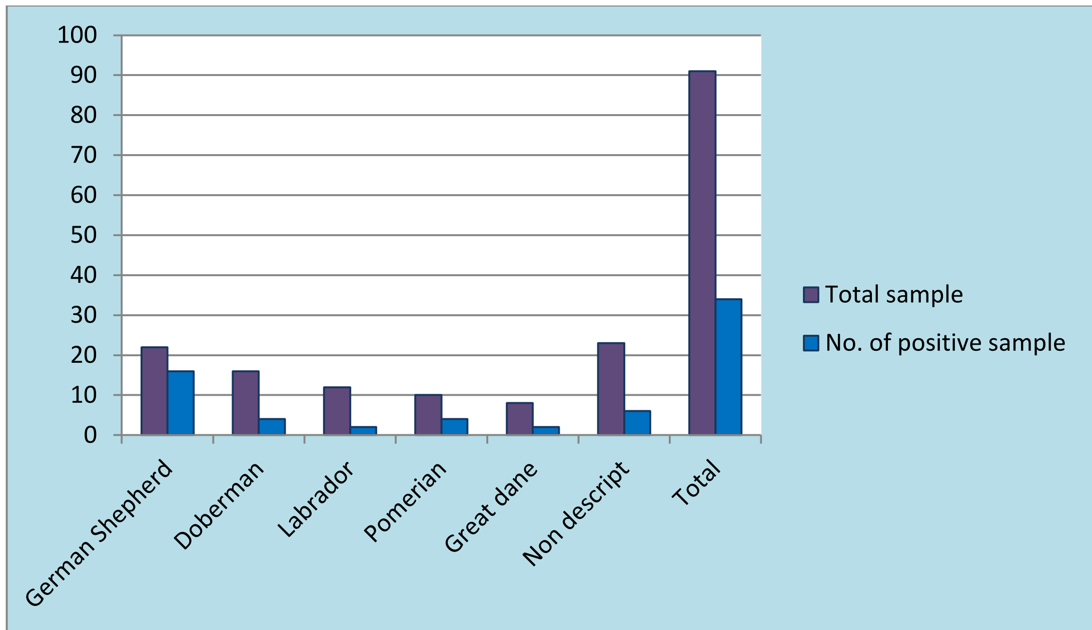

Fig. 7: Breed-Wise Prevalence of Canine Parvovirus by Antigen detection kit

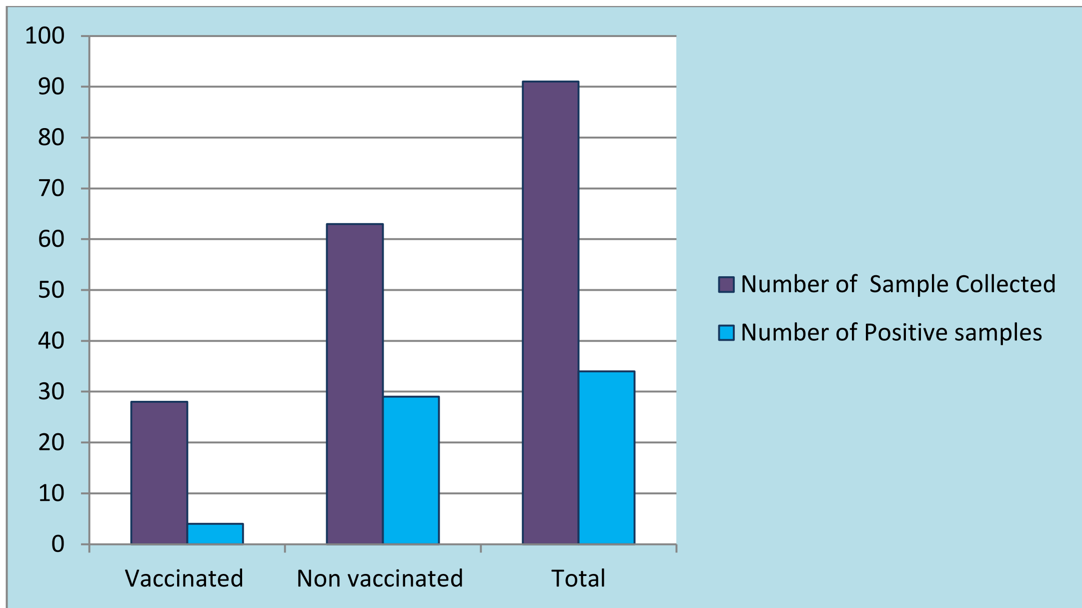

Fig. 8: Prevalence of Canine Parvovirus by Antigen detection kit based on the vaccination status

(Fig.8).Similar result found to Vakili *et al.* (2014) who reported,out of 50 samples tested, 39 (78%)were positive below the age of 6 months.Also, the similar result of sex-wise prevalence in the present study agreed with Vakili *et al.*(2014) reported increasing incidences with male (62%) dogs. Vaccinated dogs (16%) are less prominent as compared to un-vaccinated (35.41%) Reddy *et al.* (2015). As per Cavalla *et al.*(2001) as low number of incidences are reported with vaccinated dogs indicated the success of the vaccination in them.

4.3. Detection of CPV-2 by polymerase chain reaction.

PCR is sensitive, rapid technique to detect the nucleic acid from various samples, PCR has been improved the accuracy of diagnosis and help to identify infectious cause of diseases.PCR has ability to synthesis millions copies of specific segments of genes.

PCR technique has been widely used for diagnosis of Canine parvovirus, as compare to haemagglutination test and other conventional diagnostic test, PCR gives most sensitive and accuracy of diagnosis.PCR has been first time used for diagnosis of canine parvovirus in Turkey.

In present study viral DNA was amplified by PCR assay using VP2 gene specific primers. Total 91 rectal swabs were collected from the dogs showed signs of diarrhoea, and hemorrhagic enteritis, gastroenteritis with pyrexia. The PCR assay for VP2 gene was first standardized to obtain the maximum amplification of partial length of 681 bp product of the gene. Collected samples were screened by PCR. DNA was extracted from faecal sample of dogs for detection of CPV by Promega DNA extraction kit.Quality of DNA was checked on Nanodrop (Eppendorf). Out of 91 faecal samples, 41 samples were found positive by PCR with product size of 681 bp.Incidence rate of canine parvovirus was found to be (45.05%) according to present study. The result is depicted in (Table 6. Plate 8). Nearly similar findings were reported by Behera *et al.* (2015) by testing 71 faecal samples about 29 (40.85%) samples recorded positive by involving PCR assay for the study of epidemiology of canine parvovirus infection in dogs located in Bhubaneswar.

Kaur *et al.* (2015) were collected 100 fecal samples from diarrheic dogs, out of these 11 samples were found positive by PCR yielding a product size of 1198 bp.

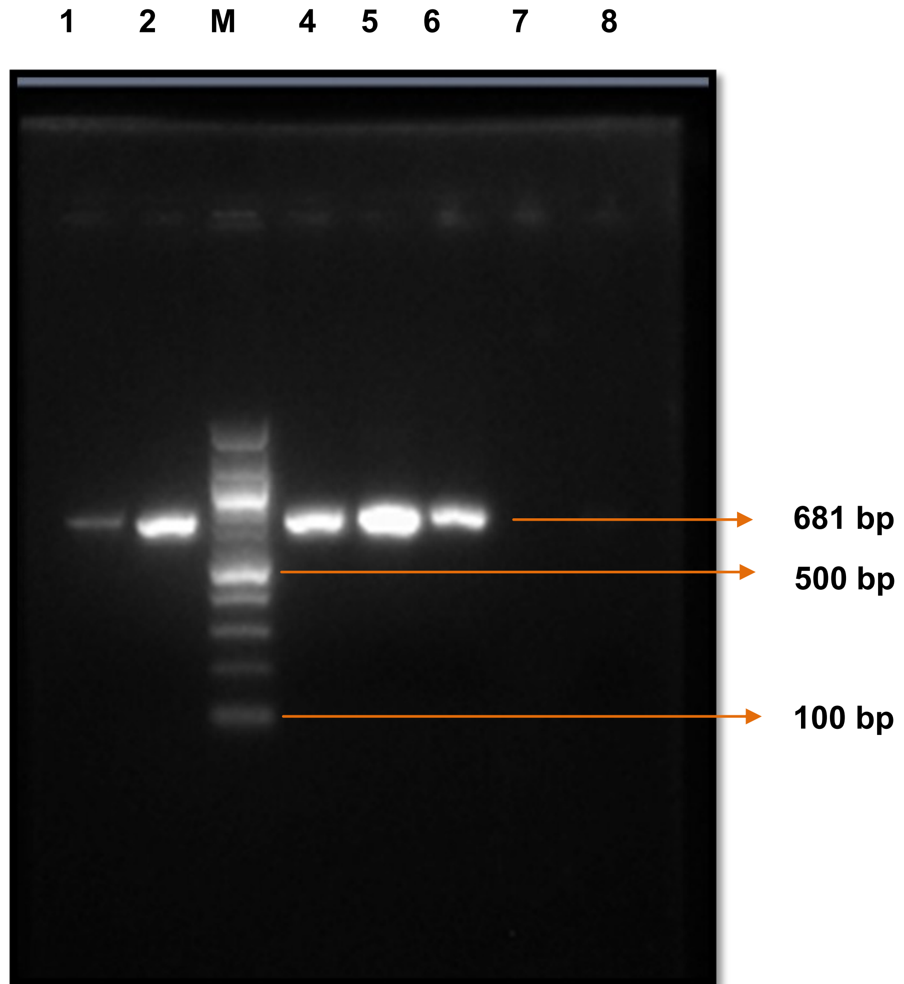

Plate 8 :Amplification of VP2 gene of Canine parvovirus-2 by PCR.

Lane 1:-Sample No 11

Lane 2:- Positive control (Vaccine)

Lane 3:-Ladder 100 bp

Lane 4:- Sample No 37

Lane 5:- Sample No 36

Lane 6:- Sample No 68

Lane 7:- Negative sample

Lane 8:- Blank

Apaa *et al.* (2016) collected 53 faecal samples from dogs, of them20 samples were found positive by PCR yielding a product of 430 and 193 bp.Kulkarni *et al.* (2016) extracted DNA'sfrom 25 fecal samples of canine parvovirus out of them 09 samples were found to be positive by PCR with band of 747 bp size.Ahmed *et al.* (2018) were collected total 40 rectal swabs from dog showing symptoms of bloody diarrhea. Out of them 33 samples were confirmed by amplified partial VP2 gene using specific primer pair Hfor/Hrev through PCR.

Table 6: Details of Age, Sex, Breed and Vaccination Status of samples positive by PCR.

Sr.No	Lab Code	PCR positive	Age	Sex	Breed	Vaccination status
1	CPV-11	+	1.5	M	German Shepherd	V
2	CPV-15	+	2	F	Labrador	NV
3	CPV-20	+	1	M	Doberman	NV
4	CPV-21	+	9	M	Labrador	NV
5	CPV-18	+	3	F	German Shepherd	V
6	CPV-36	+	3	M	German Shepherd	NV
7	CPV-37	+	1	M	Doberman	NV
8	CPV-41	+	1	F	Doberman	NV
9	CPV-55	+	2.5	M	Labrador	NV
10	CPV-54	+	1	F	German Shepherd	NV
11	CPV-60	+	2	M	Great dane	V
12	CPV-62	+	1.5	M	Doberman	NV
13	CPV-68	+	2.5	F	Great dane	NV
14	CPV-72	+	3	M	German Shepherd	NV
15	CPV-29	+	4.5	F	German Shepherd	V
16	CPV-43	+	3	M	Non descript	NV

17	CPV-25	+	3	M	Doberman	NV
18	CPV-8	+	3	F	German Shepherd	NV
19	CPV-85	+	5.5	M	Non descript	NV
20	CPV-32	+	3	M	Doberman	NV
21	CPV-61	+	3	F	Non descript	NV
22	CPV-59	+	6	M	German Shepherd	NV
23	CPV-26	+	6	F	Pomerian	V
24	CPV-78	+	3.5	M	Non descript	NV
25	CPV-84	+	1.5	F	German Shepherd	NV
26	CPV-81	+	1	M	Non descript	NV
27	CPV-75	+	2	M	Doberman	NV
28	CPV-42	+	2	F	Pomerian	NV
29	CPV-12	+	2	M	Non descript	V
30	CPV-58	+	4.5	M	German Shepherd	NV
31	CPV-5	+	3	M	German Shepherd	V
32	CPV-7	+	4	F	Pomerian	NV
33	CPV-3	+	1.5	M	German Shepherd	V
34	CPV-77	+	4	F	Non descript	NV
35	CPV-64	+	5	M	German Shepherd	NV
36	CPV-67	+	5.5	F	Pomerian	V
37	CPV-88	+	4	M	German Shepherd	NV
38	CPV-59	+	4	M	German Shepherd	NV
39	CPV-30	+	1.5	M	Labrador	NV
40	CPV-23	+	6	M	Pomerian	NV
41	CPV-17	+	3.5	F	German Shepherd	-

4.3.1. <u>Age, sex, breed vaccination status wise prevalence of CPV-by PCR.</u>

In the present study, total 41 PCR positive faecal samples were distributed according to age, sex, breed and vaccination status. Age wise distribution showed that maximum positive samples belong to age group of 0-3 (57.77%) followed by 4-6 (35.89%) and then 6-9 (14.28%) (Fig 9). Highest prevalence was observed in male dog (49.05%) (Fig 10).As per breed study highest occurrence reported in German shepherd (72.72%), followed by Pomerian (50%), Doberman (43.75%). (Fig 11).Eight samples were positive with prevalence of (28.57%) in vaccinated and (52.83%) in non-vaccinated dogs. The results are shown in (Fig12) Thus; maximum animals affected by CPV were of 0-3 months of age similar results were also documented by (Biswas *et al.*2006, and Singh *et al.*2013) who mentioned the severity of the CPV in young animals. Result of sex-wise prevalence in the present study was similar to the report given by Khan *et al.*(2006) and Srinivas *et al.* (2013) who stated increased prevalence in male for the CPV infection.Behera *et al.*(2015),The high prevalence of CPV in male dogs might be due to more chance of exposure due to certain behavioral pattern and selective preference of keeping male dogs by pet owners. Regarding results of breed wise prevalence in the present study was similar with Singh *et al.* (2013) who stated that Labrador, Pomeranian and German shepherd breeds are more prone to CPV infection.

The PCR result of vaccination status of the dogs reveled some of the vaccinated dogs were positive for CPV by PCR indicating that it might be possible, the CPV presence in the vaccinated dogs cannot be ruled out. However history could not be obtained for possible causes of vaccination failure, faulty vaccination and interference of maternal immunity reported by (Decaro and Buonavoglia., 2012 and Chinchkar *et al.*,2014).There are reports by Nigerian Veterinarians that the canine parvovirus is common in vaccinated dogs (Shima *et al.*,2015).

4.4. Detection of CPV by Conventional and Molecular diagnostic tests.

Global spread of the parvoviral infection putting up the global concern. To add up severity numbers of clinical signs are quite common to other enteric diseases and hence put up a major challenge to detect it urgently. It is understood that regularly used conventional methods such as EM and virus

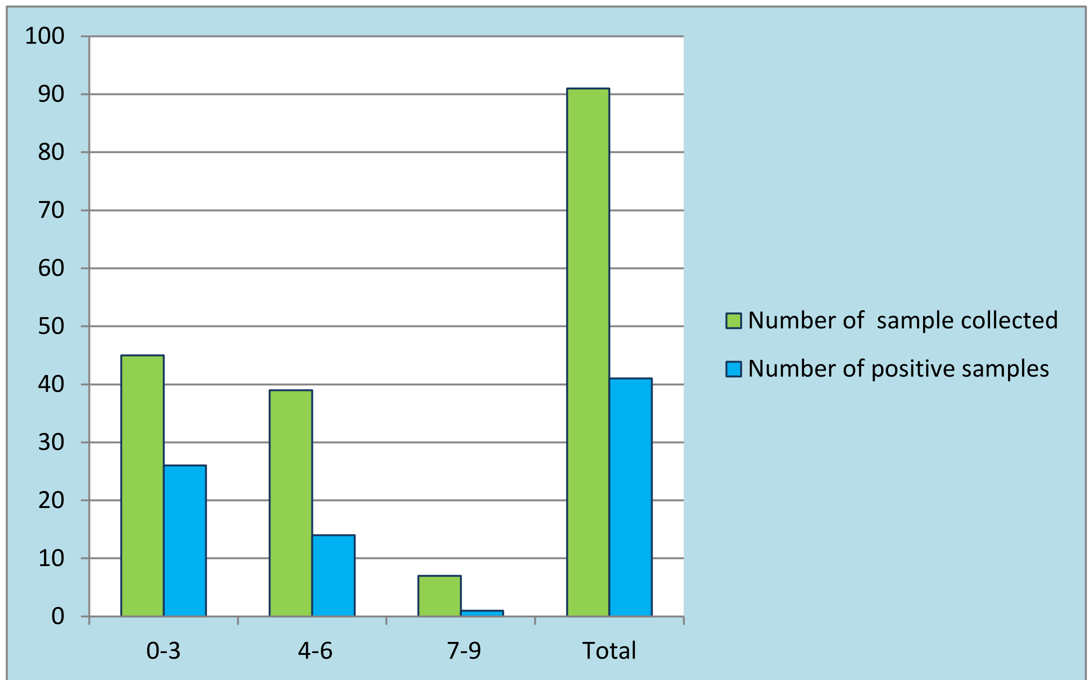

Fig. 9: Age-wise status of Canine Parvovirus by PCR

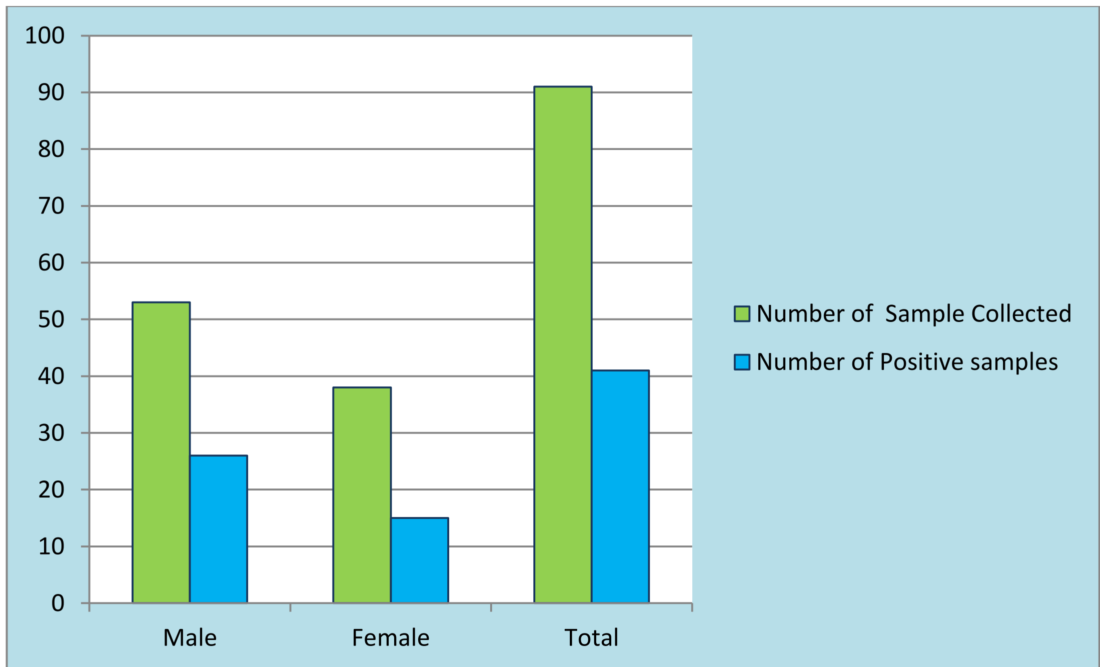

Fig. 10 : Sex-Wise distribution of Canine Parvovirus by PCR

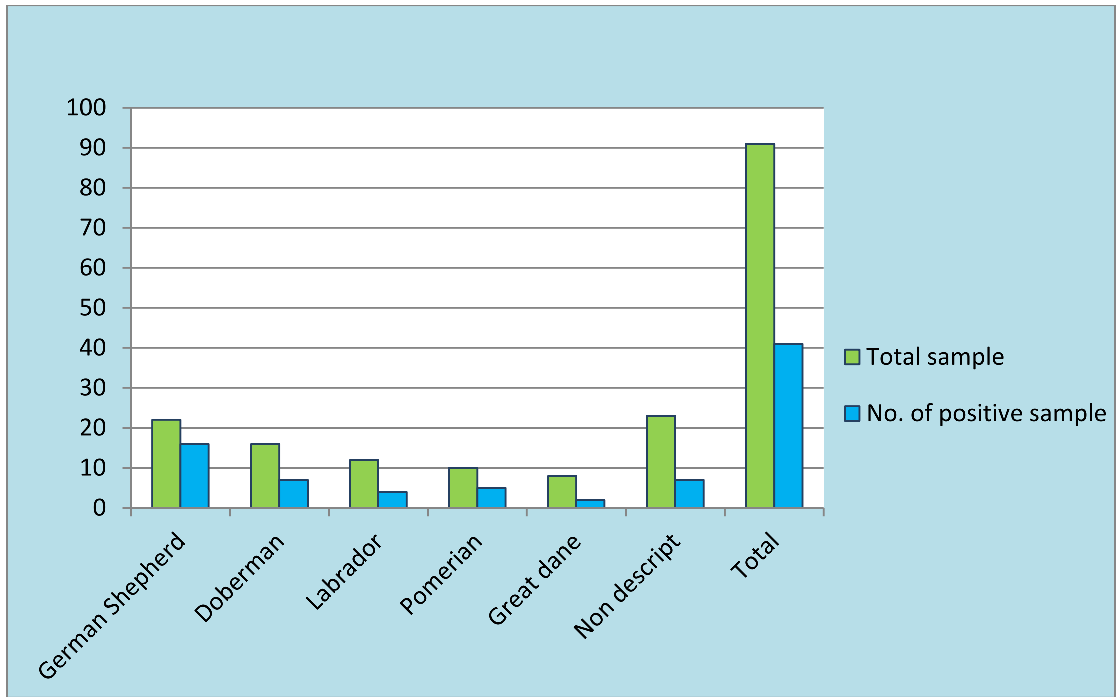

Fig. 11: Breed-Wise Prevalence of Canine Parvovirus by PCR

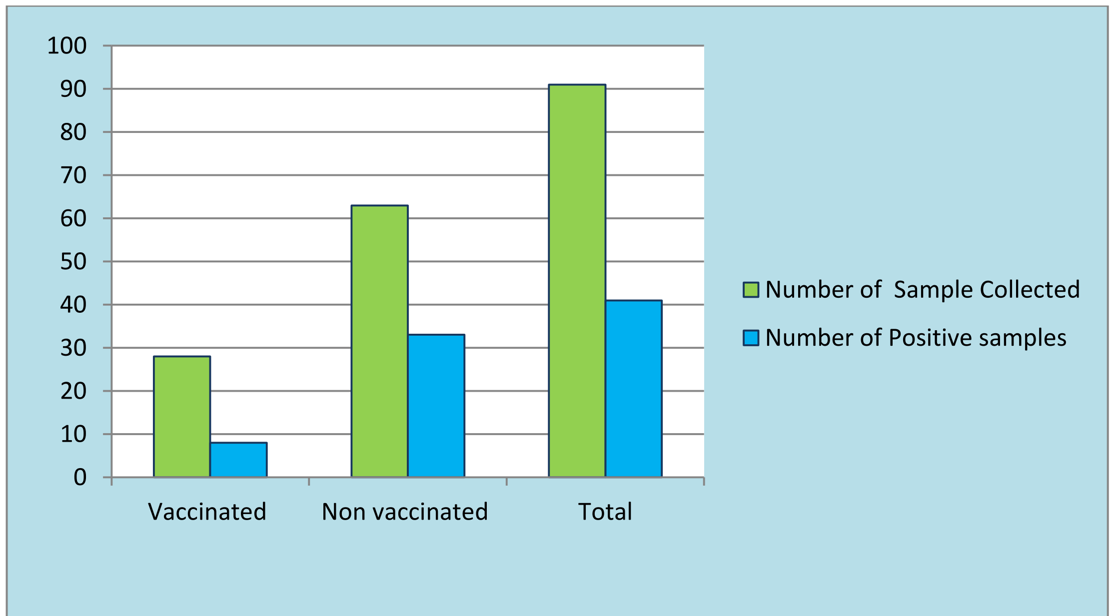

Figure. 12: Prevalence of Canine Parvovirus by PCR based on the vaccination status

isolation remain affected by the low sensitivity, time consuming approach and relatively expensive (Esfandiari, and Klingeborn.,2000, Desario *et al.,* 2005 and Cho *et al.,*2006). It is also known that serological tests by involving antibody remain successful but generally fail to detect the acute infection. Another test known as HA HI tests although remain simple but suffered from low sensitivity and demand the supply of fresh erythrocytes. (Desario *et al.,*2005 and Silva *et al*., 2013). In comparison to many failures, now molecular markers detection by PCR showcase its defined specificity (Subhashini *et al.,* 1997) and sensitivity Sakulwira *et al,* (2001) compared to used conventional antigens or other antibody-based methods. However, this test demand expensive machines and reagents and hence applied at limited places (Mohyedini *et al.,* 2013). Among them, IC based canine fecal antigen test kits represent itself the fastest kit to detect parvoviral infection and also remains sensitive, simple and rapid in performance (Vakili *et al*., 2014).

However, the necessity of expensive equipment and reagents restricts its use as a field level test (Mohyedini *et al.,* 2013). The most rapid method for diagnosis of parvoviral infections in practice is IC based canine fecal antigen test kits which are sensitive, simple, and rapid (Vakili *et al*., 2014).The test protocol is easy and does not demand special equipment (Cho *et al.,* 2006; Esfandiari and Klingeborn .,2000; and Mohyedini *et al*., 2013).

In the present study of the 91 faecal samples subjected to HA, antigen detection kit of CPV and PCR to compare the sensitivity of these tests. Out of them 22 (23.91%) samples were found positive by HA. 34 (37.36%) samples were found positive by antigen detection kit and 41 (45.05%) samples were found positive by PCR. shown in (Table 7 Fig 13)

Our results showed that PCR was more sensitive than HA and antigen detection kit.The result agreed with Vakili *et al.* (2014) who involved PCR and immunochromatography assay for detection of Canine parvovirus during hemorrhagic gastroenteritis. They reported 50 rectal swabs testing, out of them 34 samples confirmed positive with immunochromatography and as expected 50 samples positive by PCR.

HA as a test certainly now proving better as far as its low cost and implementation is concerned. As in this study, a very poor correlation between

HA and PCR method as they observed many positive viral DNA in a HA negative samples. This lack in sensitivity may be due to the absence of HA activity in CPV strains (Parrish *et al.,* 1988) or it may be possible that higher level of viral titres are demanded to produce HA and also in the fact that presence of specific antibodies in the intestinal luman majorly inconsistency most of the CPV virions, and these reducing or preventing Parvoviral binding to Erythrocyte (Decaro *et al.,* 2005; Descairo *et al.,* 2005). It is also reported that unrelated outputs of results are may be because of low HA titre (32-64) of the sample supplied.

As per Decaro *et al.,* (2010) EIA, HA and PCR remain sensitive to the all types of CPV including CPV-2C resultant of genetic variation and finds itself capable of checking even with genomic changes. It is ascertained by preliminary screening where 23-33% of dogs with negative result but symptomatically positive cases could be further checked by more sensitive PCR technique to improvise the CPV diagnosis. In a further check, possibility of mutation at a binding region of primer/probe regions needs to be the regular practice to avoid skips (Hong *et al.,* 2007).

Table 7: Details of detection of CPV by conventional and molecular diagnostic tests.

Test	HA	Antigen detection kit	PCR
Number of Positive samples	22 (23.91%)	34 (35.86%)	41 (45.05%)
Number of faecal samples tested	91	91	91

4.5. Composition of antigen detection kit with PCR:

Dogs infected with Parvovirusposing a global threat: As clinical sign is common with other enteric diseases it demands rapid diagnosis conditions and protocols to initiate the precise treatment. Among many test, rapid immunochromatography based canine faecal antigen test remains sensitive, simple and rapid. (Vakili *et al.,* 2014).In the current study, where antigen-based kit was detecting moderate positive cases when compared to PCR. Hence any antigen detection kit sensitivity possibly remains dependant on viral load

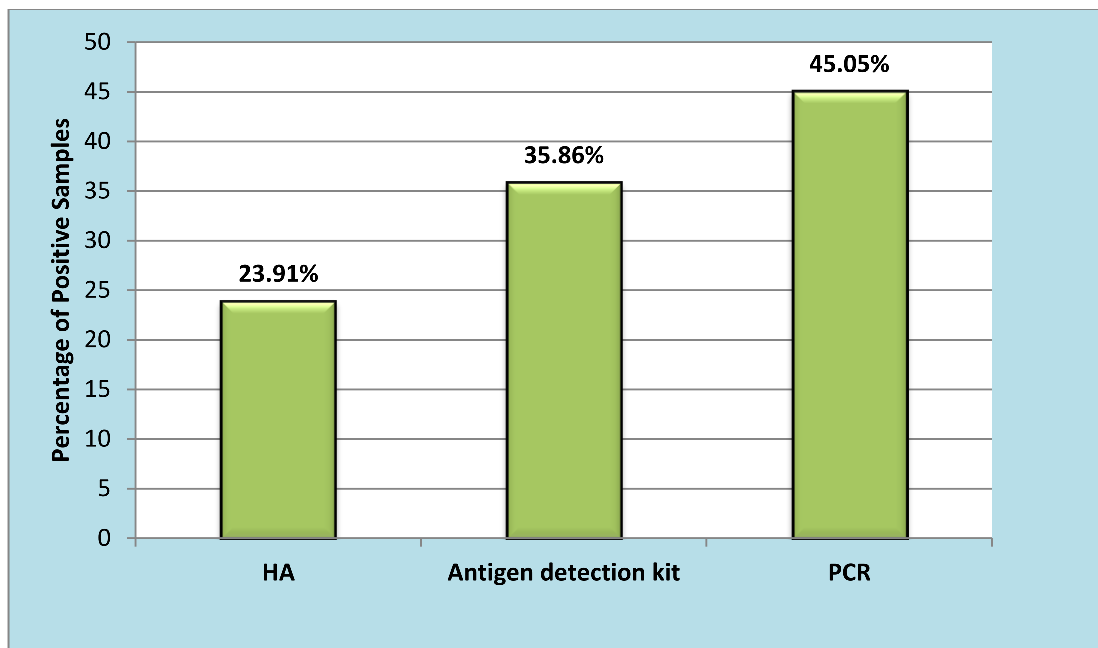

Fig. 13: Comparison of CPV-2 positive samples by HA,Antigen detection kit and PCR.

available in sample (Schimtz and Coenen., 2009).The result is represented in (Table 8).

By using combination of tests, it can be evaluated with number of different sample sets which could be involved in designing strategy for CPV identification in dogs. In the current study, when LFA test was combined with PCR, lower number of positive cases than individual test was recorded. Here two LFA positive samples failed to remain positive in PCR test. The possibility of failure in PCR could be related with inhibitory content in faecal matter. This result remains comprehensive with findings of Mochizuki *et al.* (1993) and Tinky *et al.* (2015) who confirmed the possibility of PCR failure due to presence of inhibitory materials in faeces and that resulted in false negative result. Instead, the negative PCR result, found to be positive for LFA test. This confirms the proper handling is prerequisite for managing sensitivity.

Table 8: Sensitivity and Specificity of antigen detection kit in relation to PCR for detection of Canine Parvovirus Antigen.

Test		PCR			Sensitivity	Specificity
		Positive	Negative	Total	78.08%	96%
Antigen detection kit	Positive	32	2	34		
	Negative	9	48	57		
Total		41	50	91		

In the present study, sensitivity and specificity of antigen detection kit (UbioquickVET kit) compared to PCR was found to be 78.08%and 96%respectively. The results agree with Vakili *et al.* (2014) who stated that PCR remnants more sensitive than test LFA. Tinky *et al.* (2015) confirmed the 92.8% specificity of PCR as compared to 72.7% obtained with immune chromatography strip test. In contrast, Esfandiani and Kinge Born. (2000) informed better specificity (98.8%) and sensitivity (100%) with immunochromatography assay or LFA. Hence in the current study use of LFA test to detect rapid screening in field level and further confirmation could be done by PCR.

4.6. Sequence analysis of VP2 gene of sample

For the sequence analysis, a sample randomly selected on the basis of amplicon of PCR, The PCR product were selected and submitted to the Triyad Genomic, Coimbatore, Tamil nadu, India for sequencing of VP2 gene. After obtaining the sequence, these were analyzed using NCBI BLAST and MEGA 6.0 software (http: //www.megasoftware. net/).The sequences of the sample were submitted to the DDBJ GenBank and the sample was assigned the GenBank accession number LC404128 for CPV -37. Phylogenetic tree was constructed by neighbor joining method by bootstrap with the branch length 0.1160.

On the basis of BLAST analysis, the samples and other vaccine strains had 36-42% homology with Canine Parvovirus. In the multiple sequence alignment, nucleotide sequences of the sample (CPV -37) and the vaccines were compared and aligned with the available VP2 nucleotide sequences in the Genbank, revealed that the samples belonged to the CPV-2b antigenic type.The nucleotide sequences of the samples and the vaccine strains were also phylogenetically analyzed with the VP2 gene sequences of the other Indian and world isolates available in the GenBank. From the phylogenetic tree including the Indian isolates JN625222, JN6253219,JN625220,JN625224,JN625221, KR703264 and EU274304 it was revealed that the sequence in the present study formed a separate node from rest of the Indian isolates indicating regional variation in the CPV isolates (Plate 9). From the phylogenetic tree including the worldisolates,HQ589342,HQ589342,FJ349320,FJ349321,FJ197846,FJ197847,KR703264,FJ197833,FJ197832,FJ197845,FJ197842,FJ197839,FJ197834,AB128923,KY348775,AB120727,AB120724,AB120720,AB120728,JN625223,AB120725,AB120721,AB120723,it was observed that all the 32 sequences analyzed in the present analysis were phylogenetically distributed into three separate bifurcation each having separate node .The sequence in the present study had 53 and 54% homology with the Argentina FJ349324,FJ349323 (CPV 2a and 2b) Canine Parvovirus sequence . Looking to the bootstrap phylogeny tree there was almost 50% sequence identity of occurrence of same possible taxonomic unit.

The present study findings of Phylogenetic analysis of VP2 gene of dog for parvovirus infection differ in respect to homology but similar type of virus strain circulating in India with the findings of Kaur *et al.* (2015) who performed sequence analysis of CPV. The PCR amplicon of VP2 gene was sequenced and

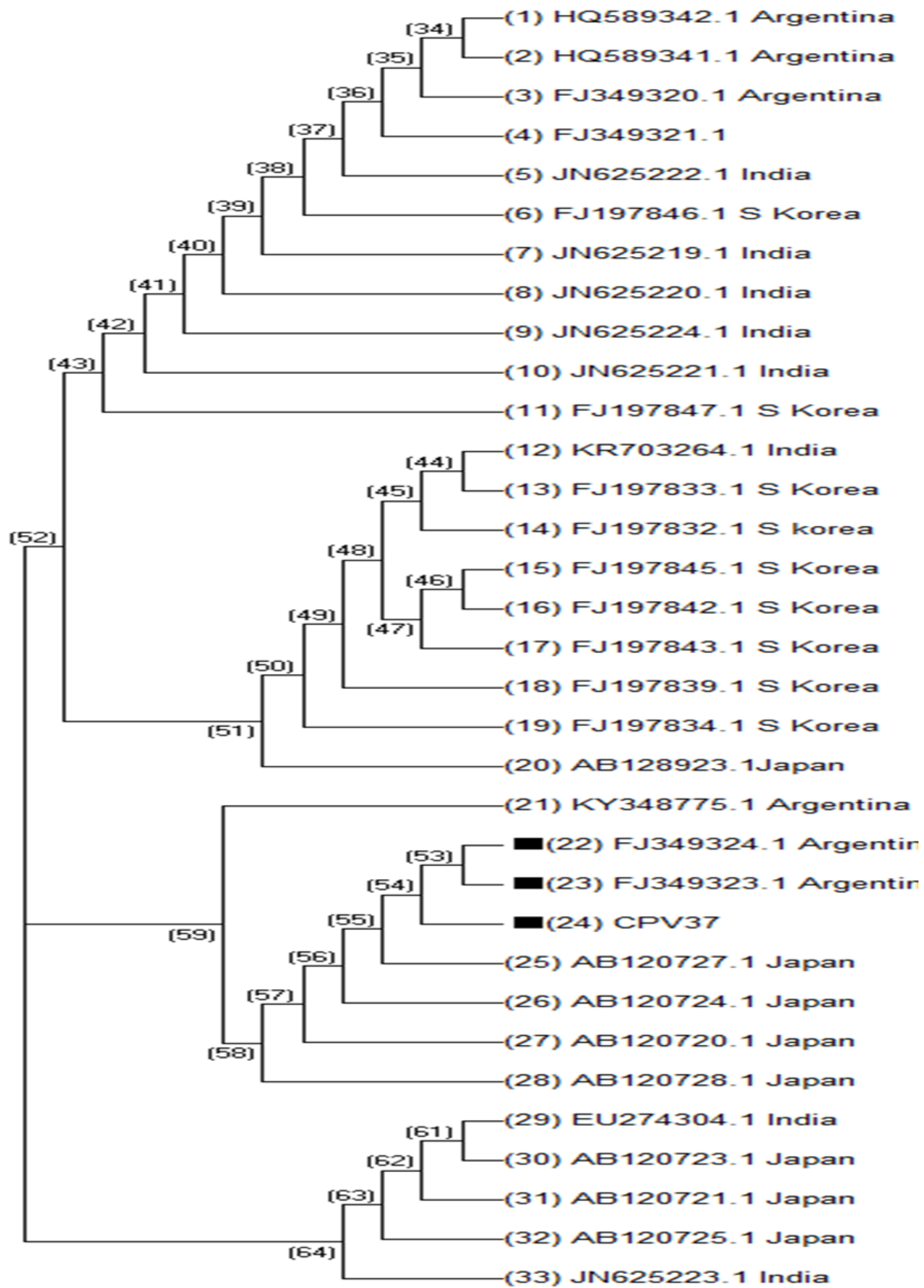

Plate 9: Phylogenetic analysis of VP2 gene of CPV-37 sample with vaccines, Indian and World isolates.

homology searched by NCBI BLAST and CLUSTAL omega. Based on the close homology four samples (P14, P44, P45 and P89) along with vaccine strains showcased 100% homology with the Query CPV. By using CLUSTAL omega nucleotide sequences of all isolates and two vaccines candidates were compared and aligned with already submitted VP2 sequences such as EU659116.1 (CPV-2), EU3103 73.2 (CPV-2a), JQ743893.1 (CPV-2b) and JF414822.1 (CPV-2c). Analysis revealed that numbers of variations was recorded at position 2773, 2816, 2817, 2885, and 3189 in the samples and two vaccines. With the CLUSTAL omega, it could be concluded that two vaccines strains belonged to CPV-2 type and related closely with CPV-2b.

Baba Sheikh *et al.* (2017) studied phylogenetic analysis using partial VP2 gene. Total eight samples analyzed for sequencing, The CPV-2 sequences originated from different continents including Asia, Europe, USA country and Africa. The VP2 sequences of CPV formed five different clusters (C1–C5). All six CPC sequences on the current study clustered within C5 branch, in together with CPV-2 of Country Egypt (Accession number KM21294). Two remaining virus sequences clustered within the C4 branch with CPV-2 sequences from Thailand, Taiwan, and China, current alignment of eight nucleotide of field virus sequences showed homology in the range 95-99%, which conclude that CPV-2b was currently the only virus circulating in Sulaimani/Iraq.

Yang *et al.* (2015) determined the nucleotide sequence of VP2 genes of QIACP1403 and QIACP1404 and framed to translation in amino acid sequences and further analyzed by molecular weight and found to be of CPV-2b type. Number of SNPs based amino acid changes were detected for VP2 gene residues of these two isolates. Phylogenetic analyses showed that these two isolates were (99.5%) having close homology with strain CPV-BM11 and remain isolated from china Dog in 2011.

The similarity rates of nucleotide identity among CPV strains were variable (78.6 to 99.16%). After phylogenetic analysis the samples was characterized as CPV -2a reported by Mohan raj. (2010), Majer-Dziedzic *et al.* (2011), Mukhopadhyay *et al.* (2014), Perez *et al.*(2014) Kulkarni *et al.*(2016) and Ahmed *et al.* (2018). Whereas, samples were characterized CPV- 2c reported by Nandi *et al.* (2010), Pinto *et al.* (2012) and Zhao *et al.* (2013). Majer-Dziedzic *et al.*(2011) reported that the mutations in the original CPV-2 strains and the

appearance of the variants (2a, 2b and 2c) were due to the particular amino acid changes predominantly in the capsid protein VP2. Perez *et al.*(2012) emphasized on the dynamic changes in CPV variants and highlighted the importance of surveillance programs to provide a better comprehension of virus epidemiology.

4.7. Isolation of CPV-2 virus in MDCK cell line:

Ability to adapt, detect and isolate parvovirus from MDCK cell lines has been attempted by inoculating one faecal sample of pups less than 6 month of age were earlier found to positive for parvovirus by PCR was selected for virus isolation in cell culture**.** After the inoculation virus get adopted in cell. The complete monolayer was obtained within 48-72 hours (plate 10).Virus inoculated in MDCK cell line and show cytopathic effect in the form of rounding of cells (plate 11) within 24 hr of post infection and after 48 hrs there was complete rounding of cell was observed, (plate 12) CPE has been marked after 72 hr in the form of clumping of cells (plate 13).The cytopathic effect remain continued after 96 hrs with detachment of cell.(plate 14) These changes were typical of parvovirus infection in MDCK cell line and were also reported by Kaur *et al.* (2015) who examined 60 samples selected for isolation, only five NPCR positive samples exhibited cytopathic effects (CPE) in the MDCK cell line. The CPE observed in the shape of rounding of cells within 24 hr, clumping of cells within 48 hr and detachment of cells within 72 hr of inoculation. Zhao *et al.* (2013) examined fecal samples, isolated the type CPV-2a from three fecal samples, among three samples, one isolate CPV-2 and with second blind passages, typical CPE as rounding, increased granularity and detached cells recorded to be appearing in the contaminated F81 cells.Kaur *et al.* (2015b),isolated canine parvovirus-2 by using MDCK cell line, samples shoe cytopathic effect in the form of rounding, clumping and detachment of cell,complet destruction of cell structure after 96 hours of PI. Brindalakshmi *et al.* (2016), isolated virus in CRFK cell line, rounding as well as degenerative changes were observed as cytopathic effect at third passage level of 72 hours post infection. Vieira *et al.* (2017) was isolated 67 out 100 (67%) CPV samples. The cytopathic effect remained associated by cell rounding and/or partial or total lysis of the formed monolayer when checked with control.

The cell culture fluid from the samples exhibiting CPE was collected to isolate the DNA. The DNA extracted from cell culture fluid of the samples

exhibiting CPE was subjected to PCR. In PCR, 681bp product were amplified which confirmed the presence of CPV and the harvested materials were preserved at -80°C.

Plate 10 : Monolayer of MDCK cell line at 10 x magnification

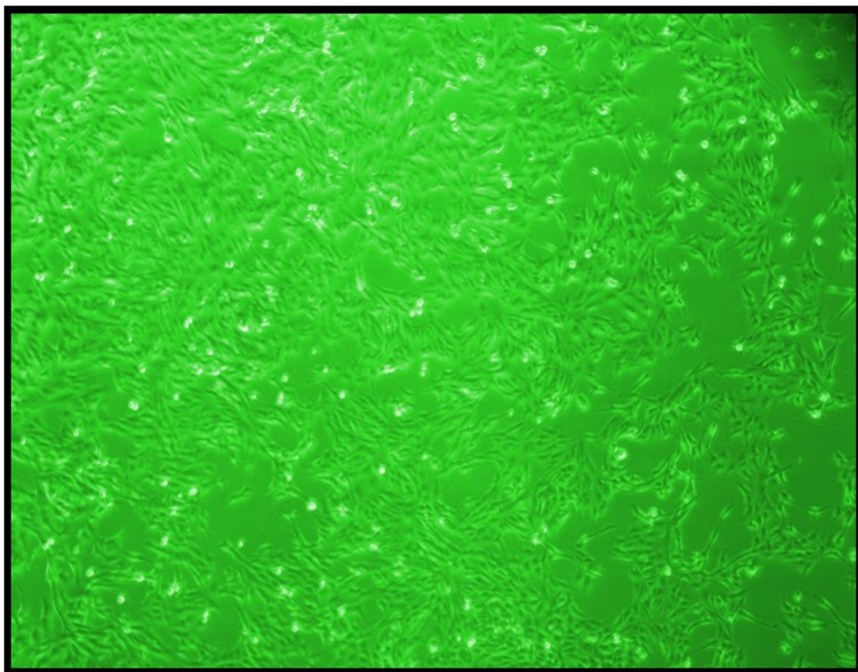

Plate 11 :Rounding of cell start at 24 hours of post-infection at 10 x magnification

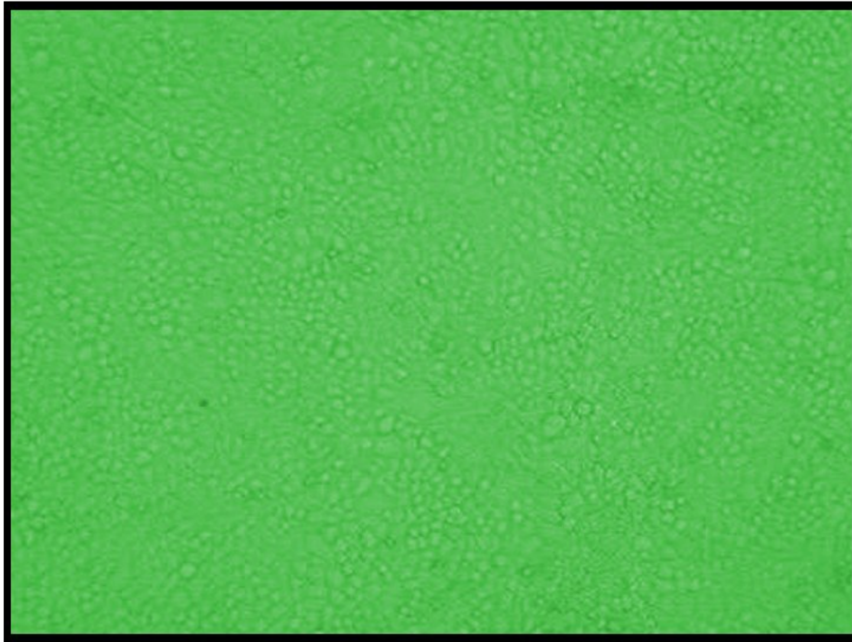

Plate 12 :Complete rounding of cells after 48 hours of post-infection at 10X magnification.

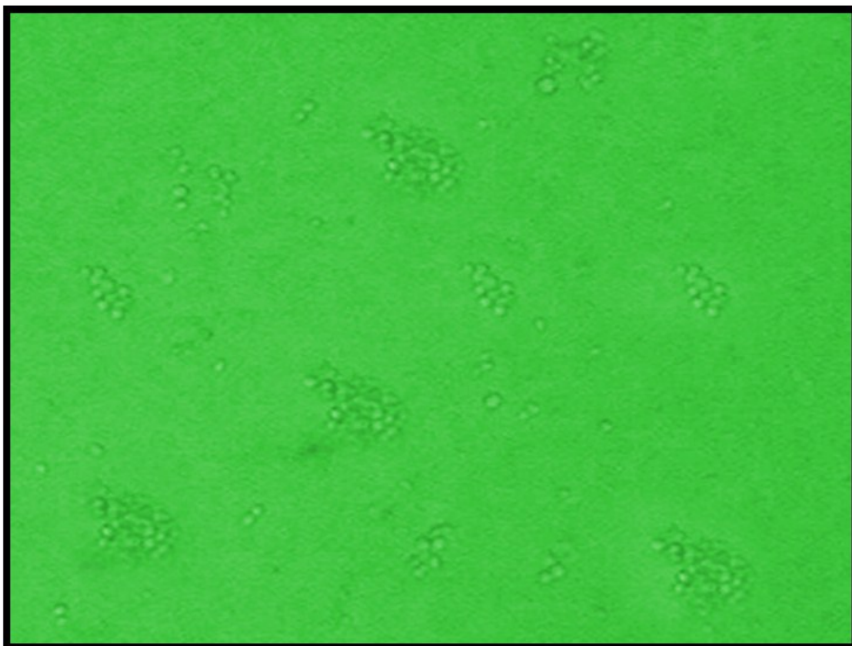

Plate 13 : Clumping of cell observed after 72 hours of post-infection at 10 x magnification

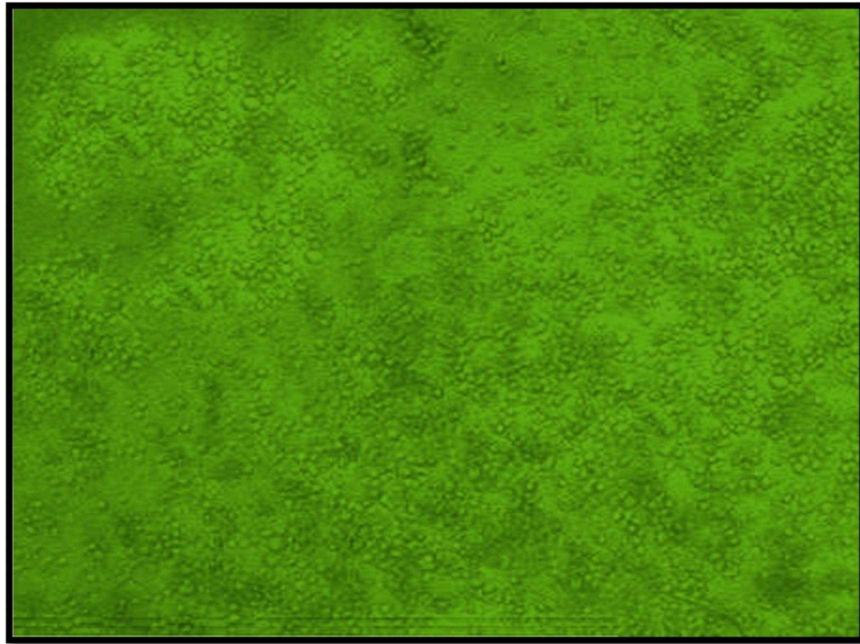

Plate 14: Detachment of cell observed after 96 hours of post-infection at 10x magnifications.

SUMMARY AND CONCLUSION

Canine parvovirus is one of the most important viral diseases among the dog population, highly contagious disease of dog causing hemorrhagic enteritis and myocarditis.CPV-2 is stable virus which mainly affecting canine population.CPV-2 spread from dog to dog by straight or incidental contact with infected feces of dog. In India, the disease was first time reported by Balu and Thangaraj in 1981.Many cases reported from vaccinated dogs.CPV is widely prevalent throughout the world and numbers of incidence have been reported from India, early diagnosis is an important aspect for preventing dog from such virus, by keeping in view, present study was tackled to study the molecular characterization of canine parvovirus-2 and different diagnostic aspect of CPV.

In the present study, total 91 samples were obtained from dog exhibiting the sign of severe gastroenteritis.Collected faecal samples were screened by haemagglutination test, Antigen detection kit using UbioquickVET kit, Detection of VP2 gene by PCR, Sequencing and Phylogenetic analysis and isolation of virus using MDCK cell line. Among the Samples (91) screened, 22 samples were established positive with HA titre>64. Highest prevalence of Canine parvovirus was observed in the age category of 0-3 (26.60%)followed by 3-6 (23.07%) and 6-9 (14.28%).It was observed that pups below the age of 6 month are most prone to CPV infection.

Sex wise prevalence showed that maximum prevalence was observed in male (30.18%) as compare to female (15.78%) dogs by HA test.In the current study, unvaccinated dogs (30.15%)were showed maximum prevalence than vaccinated dogs (17.8%), but some vaccinated dogs were also positive for CPV infection by HA test due to antigenic variant.

Total 91 faecal samples were screened by using UbioquickVET antigen detection kit,among these 34(35.86%) samples were found positive by kit. Highest prevalence of CPV was observed in the age group 0-3 (51.11%) followed by 3-6 (48.71%) and 6-9 (14.28%).Male (60.37%) showed high prevalence than female (28.94%).Four samples were found positive in vaccinated dogs with prevalence (14.28%) and in non-vaccinated dog prevalence was observed

(46.03%).Twelve faecal samples which were negative by HA test were found positive by Antigen detection kit.(UbioquickVET).

Further samples were processed for DNA extraction and subjected for PCR for detection of VP2 gene. Out of 91 faecal samples 41(45.05%) samples were found positive with uniform band of 681 bp.By PCR, maximum prevalence observed in age group of 0-3 (57.77%)and 3-6 (35.89%).Sex wise male (49.05%) were more susceptible for CPV infection than female (39.46%)dogs and unvaccinated (52.38%) dog showed highest prevalence rate than vaccinated (28.57%) dogs, indicated that new antigenic strain was circulating and due to these there was failure of vaccination .Among all these test ,Breed wise examination of data indicated that highest currency in German shepherd (72.72%), followed by Pomerian (50%) and Doberman (43.75%).

PCR was found to be highest sensitivity detecting (45.05%) as positive followed by antigen detection kit (35.86%) and HA (23.91%).PCR was further compared with the Antigen detection Kit for detection of CPV infection with respect to sensitivity and specificity. The antigen detection kit show moderate sensitivity and specificity as compared to PCR. The sensitivity and specificity of PCR was 78.08% and 96%.

After amplifying VP2 gene by specific primer, further PCR product was subjected for sequenced commercially and obtained sequence data was analyzed using bioinformatics tool such as NCBI BLAST and phylogenetic analysis was carried out using MEGA 6 software. The sequences of the sample were submitted to the DDBJ Genbank and the sample was assigned the Genbank accession number LC404128 for CPV -37sample which belonged to the CPV-2bantigenic type.

On the basis of BLAST analysis,In the multiple sequence alignment, nucleotide sequences of sample (CPV -37) and the vaccines were compared and aligned with the available VP2 nucleotide sequences in the Genbank.Phylogenetic analysis revealed that the sample and other vaccine strains has36-42% homology.The nucleotide sequences of the samples and the vaccine strains were also phylogenetically analyzed with the VP2 gene sequences of the other Indian and world isolates available in the GenBank.It revealed that the sequence in the current study formed a separate cluster from

rest of the Indian isolates indicating regional variation in the CPV isolates.It was observed that all the 32 sequences analyzed in the present study were found that isolates were distributed into three separate bifurcation, each having separate node .The sequence in the present study has 53 and 54% homology with the Argentina FJ349324,FJ349323 (CPV 2a and 2b) of Canine Parvovirus sequence.

Among all PCR positive samples, one sample randomly selected for virus isolation in MDCK cell line, virus gets adopted after 3 passages. Characteristic cytopathic effect was noticed after 24 hours of post infection in shape of rounding of cell, followed by complete rounding of cell after 48 hours, clumping in 72 hours of post infection and eventually detachment of cell after 96 hours.

Thus it can be concluded from the present study that:

Conclusion

1. Canine faecal samples were screened by Haemagglutination test (HA), antigen detection kit and PCR, revealed that 23.91%, 35.86% and 45.05% cases positive for CPV-2.
2. Highest incidence of canine parvovirus-2 was found in pups' upto the age of 3 month.
3. There was significance difference between sexes with respect to susceptibility to CPV-2 infection. Males were found to be more prone to CPV-2 infection than female.
4. The CPV-2 positive cases were also found in dogs that had been vaccinated for the virus.
5. German shepard (72.72%) was found to be more prone for CPV-2 infection followed by Pomeranian (50%) Doberman (43.75%) and Non-descript (30.43%).
6. The prevalence of CPV-2 based on the age, sex and breed was similar in HA, Antigen detection Kit and PCR.
7. PCR is more sensitive than HA and antigen detection kit.
8. The Sensitivity and specificity of PCR with other diagnosis test was established to be 78.08%and 96%.

9. PCR with highest sensitivity was taken as a gold standard to relate the finding of CPV-2 with other diagnostic test.
10. From the sequence and phylogenetic analysis, it was concluded that the isolates of Nagpur region showing 53-54%homology with the isolates from the Argentina.
11. Partial length VP2 gene sequence analysis revealed the sample CPV -37 (Accession number-LC404128) belonged to CPV-2b.
12. One positive sample was used for infecting MDCK cell line showed significance cytopathic effect (CPE) in shape of rounding (48 hours), clumping (72 hours) and detachment (96 hours) of post infection was observed.

BIBLIOGRAPHY

Ahmed.N, A.Riaz,Z.Zubair,M. Saqib,S.Ijaz, Muhammad Shah Nawaz-Ul-Rehman,A. Al-Qahtani and M.Mubin (2018) Molecular analysis of partial VP-2 gene amplified from rectal swab samples of diarrheic dogs in Pakistan confirms the circulation of canine parvovirus genetic variant CPV-2a and detects sequences of feline panleukopenia virus (FPV).Virol. J. 15:45.

Al-Bayati, H. A., M. Odishosh and H. A. Majeed (2010) Detection of canine parvovirus in Iraq by using rapid antigen test kit and haemagglutination –inhibition test. Al Anbar J.Vety. Sci. 3: 17–23.

Apaa, T.T., J.M.Daly,R.E.Tarlinton (2016) Canine parvovirus (CPV-2) variants circulating in Nigerian dogs. Vet. Rec. Open. 3: 1-4.

Appel M.J.G,B.J. Cooper, H.Greisen and L.E. Carmichael (1978) Status report: canine viral enteritis. J. Am. Vet. Med. Assoc. 173: 1516-1518.

Baba Sheikh, M. O., P. M. A. Rashid, A. S. Marouf, Z. H. Raheem, S. Manjunath & S. C. Janga (2017)Molecular typing of canine parvovirus from Sulaimani, Iraq and phylogenetic analysis using partial VP2 gene. Bulg. J. Vet. Med.20(3):225-235.

Badgett, M. R.,A. Alexandra, L. E.Carmichael, C.R.Parrish and J.J. Bull (2002) Evolutionary dynamics of viral attenuation. J.Virol.76(20): 10524-10529.

Balu, P. A and T.M.Thangaraj (1981) Canine viral gastroenteritis- A clinical report. Ind.J.Vety. Med. 1:73.

Banja, B. K.,H.K. Panda,S.K. Ray and P.K. Das (2002b) Epizootilogical status of canine viral haemorrhagic gastro enteritis in Bhubaneswar. Ind. Vety.J 79:850 – 851.

Banja, B.K.,N. Sahoo,P.K.Das, P.Swin and H.K.Panda (2002) Comparison of different laboratory tests for diagnosis of parvo and corona viral infections in dogs. Ind.Vety. J 79 : 425-428.

Behera, M., S.K.Panda,P.K. Sahoo, A.P.Acharya, R.C.Patra,S. Das and S.Pati (2015) Epidemiological study of canine parvovirus infection in and around Bhubaneswar, Odisha, India. Vety.World. 8: 33.

Berns, K. I (1990) Parvoviridae and their replication.Virology. 2: 1743-63.

Binn, L. N., B.C.Lazar,G.A.Eddy and M.Kajima (1970) Recovery and characterization of a minute virus of canines. Infection and Immunity 1:503-08.

Biswas, S., P. J. Das, S. K. Ghosh, N. R. Pradhan (2006) Detection of canine parvovirus (CPV) DNA by polymerase chain reaction and its prevalence in dogs in and around Kolkata, West Bengal. Ind. J. Anim. Sci. 76: 324-325.

Buonavoglia, C., V.Martella , A.Pratelli ,M. Tempesta , A.Cavalli ,D. Buonavoglia , G.Bozzo , G.Ella ,N. Decaro and L. Carmichael (2001) Evidence for evolution of canine parvovirus type 2 in Italy. J. Gen. Virol. 82(12): 3021-25.

Brindhalakshmi,B.H.K.Mukhopadhyay,P.XAntony,J.Thanislass , P.Vijayalakshmi (2016) Isolation and Molecular Characterization of Canine and Feline Parvovirus Strains - An Updated Review. J Dairy Vet Anim Res 3(5): 00093.

Carmichael L. E and L.N.Binn (1981) New enteric viruses in the dogs. Advance Vety. Sci. and Comparative Med. 25: 37.

Carmichael, L. E., J.C.Joubert and R.V. Pollock (1980) Haemaglutination by canine parvovirus: serological studies and diagnostic applications. American J. of Vety. Res. 41:784-91.

Carter, G. R and D.J.(2006) “Parvoviridae”. A Concise Review of Veterinary Virology.http://www.ivis.org/advances/Carte/Part2Chapter 9.

Cavalla, A.,G. Bozzo,N. Drecaro, A.Tinelli, A.Aliberti, D.Buonavoglia (2001) Characterization of a canine parvovirus strain isolated from an adult dog.New Microbiol. 24: 239-242.

Chinchkar, S. R., B.M. Subramanian, H.Naidu, D. Thiagarajan, V.Cho, H.S., Kang, J.I. and Park, N.Y (2006) Detection of canine parvovirus in

fecal samples using loop-mediated isothermal amplification. J. Vet. Diagn. Invest. 18: 81-84.

Chinchkar, S. R., B.M. Subramanian, H.Naidu, D. Thiagarajan, V.Cho, H.S., Kang, J.I. and Park, N.Y (2014) Canine Parvovirus Isolates of India and the Relevance of Canine Parvovirus Type-2 Vaccines. J.adv. Vety. Res.4.34-41.

Cho, H.S.,J.Kang,N.Y. Park(2006) Detection of canine parvovirus in fecal samples using loop-mediated isothermal amplification. J. Vet. Diagn. Invest. 18:81-84.

Chollom, S.C., H.G.Ularamu, T.Y. Woma, O.O Owolodun, P.D.Luka, A.R. Jambol, O.O.Nwankiti and D.Z. Egah (2012) Detection of canine parvovirus 2: A comparison of conventional polymerase chain reaction and haemagglutination test. Afri.J.Micro.Res. 6(21):4592-4596.

Cotmore, S. F and P. Tattersall (1987) The autonomously replicating parvoviruses of vertebrates. Adv. Virus Res. 33:91-174.

De Oliveira P.S.B.,J.F. Cargnelutti, E.K.Masuda,R.A. Fighera, G.D.Kommers, M.C.Silva, R.Weible and E.F. Flores(2018) Epidemiological, clinical and pathological features of canine parvovirus 2c infection in dogs from southern Brazil. Pesq. Vet. Bras. 38(1):113-118.

Decaro, N.and C.Buonavoglia (2012) Canine parvovirus-A review of epidemiological and diagnostic aspects with emphasis on type 2c.Vety. Microbiol.155 : 1-12.

Decaro, N., C.Desario, F.Amorisco,M. Losurdo, G.Elia , A.Parisi , G.Ventrella , V.Martella , C.Buonavoglia (2013) Detection of a canine parvovirus type 2c with a non-coding mutation and its implications for molecular characterisation. Vety. J. 196:55557.

Decaro, N., G.Elia, M.Campolo,C. Desario,M.S. Lucente,A.L. Bellacicco and C.Bounavoglia (2005a) New approaches for the molecular characterization of canine parvovirus type 2 strains. J. Vety. Medi.B. 52:316-19.

Decaro, N., C.Desario,M.Campolo,G. Elia,V.Martella,D.Ricci,E.Lorusso and C.Buonavoglia (2005b) Clinical and virological findings in pups naturally infected by canine parvovirus type 2 Glu-426 mutant. J. Vety. Diagnostic Investigation.17:133-138.

Decaro, N., G.Elia, V.Martella, C.Desario,M.Campolo,L.D.Trani,E.Tarsitano, M.Tempesta and C.Buonavoglia (2005) A real-time PCR assay for rapid detection and quantitation of canine parvovirus type 2 in the feces of dogs. Vet. Microbiol. 105: 19–28.

Decaro, N.,C. Desario, G.Elia , V.Martella, V.Mari , A.Lavazza, M.Nardi and C.Buonavoglia(2008) Evidence for immunization failure in vaccinated adult dogs infected with canine parvovirus type 2c. New Microbiol. 31:125-30.

Decaro, N.,C. Desario,M.J.Beall, A.Cavalli, M.Campolo,A.A.Dimarco, F.Amorisco, M.L.Colaianni and C.Buonavoglia (2010) Detection of canine parvovirus type 2c by a commercially available in-house rapid test. Vet. J. 184: 373–375.

Decaro, N.,G. Elia ,C. Desario , S.Roperto , V.Martella,M. Campolo , A.Lorusso , A.Cavalli and C.Buonavoglia(2006) A minor groove binder probe real time PCR assay for discrimination between type-2 based vaccines and field strains of canine parvovirus. J. Virological Methods. 136: 65-70.

Decaro, N.,G. Elia,V.Martella,M.Campolo,C.Desario,M.Camero (2006) Characterisation of the canine parvovirus type 2 variants using minor groove binder probe technology. J. Virol. Methods.133:92–9.

Decaro, N., C.Desario,G. Elia,V. Martella, V.Mari ,A. Lavazza, M.Nardi ,C.Buona voglia (2008) Evidence for immunization failure in vaccinated adult dogs infected with canine parvovirus type 2c.New Microbiol. 31: 125-130.

Deepa and Saseendranath (2002) Incidence of canine parvoviral infection in immunized dogs. Ind.Vety.J.79: 643-644.

Desario, C., N.Decaro, M.Campolo,A. Cavalli, F.Cirone, G.Elia,V. Martella, E.Lorusso, M.Camero and C.Buonavoglia (2005) Canine parvovirus

infection: Which diagnostic test for virus. J. Virol. Methods. 126(1-2): 179-185.

Esfandiari, J. and B.Klingeborn (2000) A comparative study of new rapid and one– step test for the detection of parvovirus in feces from dogs, cats, and mink. J. Vet. Med. Infect. Dis., Vet. Public Hlth. 47(2): 145-153.

Folitse R.D, D.Kodie, E.Amemor (2018) Detection Of Canine Parvovirus Antigen In Dogs In Kumasi, Ghana. Afri. J.Inf. Dis. 12(1):28-32.

Folitse,R.D, D.O Kodie, E. Amemo, D. Dei, W. Tasiame, V. Burimuah,B.O Emikpe (2017) Detection Of Canine Parvovirus Antigen In Dogs In Kumasi, Ghana. Afr., J. Infect. Dis. 12 (1): 28-32.

Greenwood, N. M., W.S.K.Chalmers,W. Baxendale and H. Thompson (1995) Comparison of isolates of canine parvovirus by restriction enzyme analysis and vaccine efficacy against field strains. Vety.Records. 136: 63-67.

Gupta, P. K., A. A. Raut, U. Dimri and A.Rai (2006) Rapid PCR-based method for detection of canine parvovirus in dog faeces. Ind. J. Virol.17(2): 78-81.

Hadi, A. F., J.S.Sardar, A. Mohammadi and A.Tamadon (2011) Characterization of Iranian isolates of canine parvovirus in samples using polymerase chain reaction assay. Iran. J. Biotech.9(1):63-68.

Hasan, K., D.Rathnamma, H.D.Narayanaswamy,S.Isloor, B.M.Chandranaik, M.A.Kshama , A.M.Elattuvalappil, S.Y.Mukartal , S.Singh ,S.Gupta, S.Krishnappa (2016) Comparative evaluation of lateral flow assay and PCR for detection of Canine parvovirus. Adv. Anim. Vet. Sci. 4(11): 580-583.

Hasan, M.M.,M.S.Jalal, M.Bayzid, M.A.M.Sharif and M.Masuduzzaman(2017) A Comparative Study On Canine Parvovirus Infection Of Dog In Bangladesh and India. Bangladesh J. Vety. Med. 14(2): 237.

Hoelzer, K. and C.R. Parrish (2010) The emergence of parvoviruses of carnivores. Vet. Res. 41:39.

Hong, C.,N. Decaro,C. Desario (2007) Occurrence of canine parvovirus type 2c in the United States. J .Vet.Diagn. Invest.19:535–539.

Hoskins (2006) Infectious diseases of dog and cat (Canine viral enteritis).Greene, 3rd edition, Saunders Company.40-45.

Houston, Doreen. M., C.S.Ribble and L.L.Head (1996) Risk factors associated with parvovirus enteritis in dogs: 283 cases (1982-1991). J. Ameri.Vety. Med. 208(4): 542-46.

Jones, T. C.,R.D. Hunt and N.W.King (1997) Veterinary Pathology. Blackwell Publishing.

Joshi, D. V., S.P.Singh,S.N. Sharma and B.J.Patel(2001) Clinic-pathology of experimental canine parvovirus infection. Ind. Vety J.Animal. Sci. 71:543-44.

Kasamatsu, H and A.Nakanishi (1998) How do animal DNA viruses get to the nucleus? Annu. Rev. Microbiol. 52:627-86.

Kaur, G., M.Chandra, P. N. Dwivedi,and N.S. Sharma (2015) Prevalence of canine parvovirus in dogs in Ludhiana, Punjab. Res. Environ. Life Sci. 8(2):157-158.

Kaur, G., M.Chandra, P.N. Dwivedi and N. S. Sharma (2015a) Isolation of Canine parvovirus with a view to identify the prevalent serotype on the basis of partial sequence analysis. Vet. World. 8(1): 52-56.

Khadilkar, M. V., P.Batra and N.M.Singhvi (1994) Canine parvovirus. J. Remount Vety. Corps.33:81.

Khan, M.A.,M. Rabbani, K.Muhammad,N. Murtaza and J.Nazir (2006) Int. J. Agric. Biol., 8: 898-900.

Khatri, R., Poonam, H.Mohan, Minakshi, C.S.Pundir (2017) Epidemiology, Pathogenesis, Diagnosis and in Treatment of Canine Parvovirus Disease Dogs: A Mini Review Abstract. J. Vet .Sci. Med. Diagn .6:3.

Kilian, E.,J.S. Suchodolski, K.Hartmann,R.S. Mueller , G.Wess , S.Unterer (2018) Long-term effects of canine parvovirus infection in dogs. PLoS ONE 13(3): e0192198.

Kulkarni,M.B (2016) Molecular characterization and phylogenetic analysis of canine parvovirus. M.V.Sc thesis submitted to Maharashtra Animal And Fishery Sciences University,Nagpur.

Kumar, M., S. Chidri and S. Nandi (2011) A sensitive method to detect canine parvoviral DNA in faecal samples by nested polymerase chain reaction. Indian J. Biotechnol. 10:183–187.

La Torre, D.D., E.Mafla, B.Puga, L.Erazo, C.Astolfi-Ferreira,A.P.Ferreira (2018) Molecular characterization of canine parvovirus variants (CPV-2a, CPV-2b, and CPV-2c) based on the *VP2* gene in affected domestic dogs in Ecuador.Vety. World. 11(4):480-487.

Lamas-Saiz, A.L.,M. Agbandje-McKenna,J.S.L. Parker, A.T.M.Wahid , C.R.Parrish and M.G.Rossmann (1996) Structural analysis of a mutation in canine parvovirus, which controls antigenicity and host range. Virology. 225:65-71.

Majer-Dziedzic, B., A.Jakubczak and J.Zietek (2011) Phylogenetic analysis of canine parvovirus CPV-2 strains and its variants isolated in Poland. Polish J. Vety. Sci.14 (3): 379-84.

Manoj Kumar, S. Chidri and S.Nandi (2010) Molecular cloning and restriction endonuclease analysisof canine parvovirus DNA amplified by polymerase chain reaction. Global Veterinaria; 4(2): 125-129.

Marulappa, S. and S.Kapil, (2009) ''Simple test for rapid detection of canine parvovirus antigen and canine parvovirus specific antibodies''.Clinical and Vaccine Immunology, 16: 127-131.

Meunier, P. C., B.J.Cooper, M.J.Appel and D.O.Slauson (1985) Pathogenesis of canine parvovirus enteritis.The importance of viraemia. Veterinary Pathology 22:60-71.

Mildbrand, M.M., Y.A.Teramoto, J.K.Collins,A. Mathys and S.Winston (1984) Rapid detection of canine parvovirus in faeces using monoclonal antibodies and enzyme-linked immunosorbent assay. Am. J. Vet. Res. 45: 2281-2284.

Mizak, B.,A. Rzezutka,J. Krol and A.Kazula(2001) Construction and immunogenic properties of DNA vaccine against canine parvovirus infection. Bulletin of the Veterinary Institute in Pulway. 45:125-39.

Mochizuki,M., M.C.San,Gabriel,H.Nakatani,M.Yoshida,and R. Harasawa(1993) Comparison of polymerase chain reaction with virus isolation and haemagglutination assays for the detection of canine parvoviruses in faecal specimens. Research in Vety. Sci. 55:60-63.

Mohan, Raj J., H.K.Mukhopadhyay, J.Thanislass,P.X. Antony,R.M. Pillai (2010) Isolation, molecular characterization and phylogenetic analysis of canine parvovirus. Infect. Genet.Evol. ;10:1237–1241.

Mohyedini, S., S.Jamshidi , S.Rafati , G.H.R.Nikbakht, A. Malmasi , Y.Taslimi, H.Akbarein (2013) Comparison of immunechromatographic rapid test with molecular method in diagnosis of canine parvovirus. Iran. J. Vet. Med. 7(1): 57-61.

Mosallanejad, B., G.M.Najaf abadi and R.Avizeh (2008) The first report of concurrent detection of canine parvovirus and corona virus in diarrheic dogs of Iran. Iranian J. Vet. Res. 9(3):284 -286.

Mukhopadhyay, H.K., S.Lakshmi Matta, S. Amsaveni, P.X. Antony, J.Thanislass and R.M. Pillai (2014) Phylogenetic analysis of canine parvovirus partial VP2 gene in India. Virus Genes. 48: 89-95.

Murphy, F.A., E.P.J.Gibbs,M. Horzinek and M.J. Studdert (1999) Veterinary Virology. 3rd ed. New York: Academic Press. 343-354.

Muthuraj, P.G.,J. Thomas,S. Verma,C. Sharma,T.K. Goswami and M. Singh (2016) Usefulness of Haemagglutination Test for Screening of Canine Parvovirus Infection in Dogs. J. Immunol. Immunopathol. 18(2): 144-147.

Mylonakis, M. E., I.Kalli, and T.S. Rallis,(2016) Canine parvoviral enteritis: an update on the clinical diagnosis, treatment, and prevention. Veterinary Medicine: Research and Reports. 7: 91–100.

Nakamura, M., Y.Tohya, T.Miyazawa,M. Mochizuki, H.T.Phung , N.H.Nguyen, L.M.Huynh , L.T.Nguyen , P.N.Nguyen , P.V.Nguyen, N.P.Nguyen and H.Akashi (2004) A novel antigenic variant of canine parvovirus from a Vietnamese dog. Arch. Virol. 149:2261-69.

Nandi, S., R. Anbuzhagan and Manoj Kumar (2010) Strain differentiation and characterization of canine parvovirus by PCR and RE mapping. Ind.J.Biotechnol. (9):38-42.

Nandi, S., V. Chander, C. Ravishankar, M.Bitw and T. K. Mahopatra (2015) Rapid and sensitive method of detection of canine parvovirus infection in dogs by polymerase chain reaction. Ind. J. Canine Practice.7(1): 1-6.

Nandi, S., R.Anbazhagan and M. Kumar (2010) Molecular characterisation of nucleotide sequence analysis of canine parvovirus strains in vaccines in India. Veterinaria Italiana 46(1):69-81.

Nandi,S.,S.Chidri, M.Kumar and R.S.Chauhan (2010a) Occurrence of canine parvovirus type 2C in the dogs with hemorrhagic enteritis in India. Res. Vet. Sci. 88:169-171.

O.I.E.Manual for diagnostic test and vaccine (1992) 2ed. Published by Office International des epizootics.

Oh, J.,G.Ha,Y.Cho,M. Kim,D.J. An, K.Hwang, Y.Lim,B.Park,B. Kang and D.Song (2006) One-step immune chromatography assay kit for detecting antibodies to canine parvovirus. Clin.Vacc.Immunol. 13(4): 520524.

Parker, J. and J.Chen,(2017) Application of next generation sequencing for the detection of human viral pathogens in clinical specimens. J. Clin. Virol.: the official publication of the Pan American Society for Clinical Virol. 86: 20–26,

Parker, J. S. L.,W.J. Murphy, D.Wang,S.J. O'Brein and C.R.Parrish (2001) Canine and feline parvoviruses can use human and feline transferrin receptors to bind, enter and infect cells. J. Virol. 75: 3896-902.

Parrish, C. R., C.F. Aquadro, M. L. Strassheim, J.F. Evermann, J. Sgro and H.O. Mohammed (1991) Rapid antigenic-type replacement and DNA sequence evolution of canine parvovirus; 65(12):6544-6552.

Parrish, C. R., (1991) Mapping specific functions in the capsid structure of canine parvovirus and feline panleukopenia virus using infectious plasmid clones. Virology. 183 (1): 195-205.

Parrish, C. R., (1999) Host range relationship and the evolution of canine parvovirus.Vety.Microbiol. 69: 29–40.

Parrish,C.R.,G.Burtonboy,L.E.Carmichael (1988)Characterization of a nonhemagglutination mutant of canine parvovirus. Virology. 163:230-232.

Parthiban, M., I.C.Kurunch, K.Divya (2012) A rapid and highly reliable field-based LAMP assay of canine parvovirus. Acta Virol. 56:71-74.

Parthiban, S., H.K.Mukhopadhyay, D.Panneer, P.X.Antony, and R.M. Pillai(2011) Isolation and Typing of Canine Parvovirus in CRFK Cell Line in Puducherry, South India. Indian J. Microbiol. 51(4): 456–460.

Pedro, C., A. Duarte, S. Gil, C. Cartaxeiro, M. Malta, S. Vieira and L. Tavares (2014) Molecular and serological surveillance of canine enteric viruses in stray dogs from Vila do Maio, Cape Verde.BMC Vet. Res. 10(91) :1-7.

Pérez, R., L.Calleros ,A. Marandino,N. Sarute, G.Iraola, S.Grecco (2014) Phylogenetic and Genome-Wide Deep-Sequencing Analyses of Canine Parvovirus Reveal Co-Infection with Field Variants and Emergence of a Recent Recombinant Strain. PLoS ONE 9(11): e111779.

Perez, R., P. Bianchi, L. Calleros, L. Francia, M.Hernandez, L. Maya, Y. Panzera, K. Sosa and S.Zoller (2012) Recent spreading of a divergent canine parvovirus type 2a (CPV-2a) strain in a CPV-2c homogenous population. Vet. Microbiol. 155: 214-219.

Phromnoi, S., R.Sinsiri and T. Sirinarumitr (2010) Expression of Recombinan VP2 Protein of Canine Parvovirus in Escherichia coli. Kasetsart Journal (Natural Science) 44: 870 -78.

Pinto, L.D., A.F.Streck, K. R. Goncalves, C.K.Souza, A. O. Corbellini,L.G. Corbellini and C. W.Canal (2012) Typing of canine parvovirus strains circulating in Brazil between 2008 and 2010. Virus Res.165:29-33.

Pratelli, A., A.Cavalli, V.Martella, M.Tempesta, N.Decaro,L.E.Carmichael and C.Bounavoglia (2001) Canine parvovirus (CPV) vaccination: comparison of neutralizing antibody responses in pups after inoculation with CPV-2 or CPV-2b modified live virus vaccine. Clin.Diagn. Lab. Immunol. 8:612-15.

Puentes, R., N.Eliopulos, R.Pérez, G.Franco,K.Sosa, P.Bianchi, A.Furtado,S.O. Hübner, P.A.Esteves (2012) Isolation and characterization of canine parvovirus type 2C (CPV-2C) from symptomatic puppies. Brazilian J. Microbiol. 43: 1005–1009.

Radostits, O.M., D.C. Blood and J.A. Henderson (2000) Veterinary Medicine 9th Ed. Bailliera Tindall, London.

Ramadass, P and T.G.A.Khader (1982) Diagnosis of canine parvovirus infection by agar gel precipitation test and fluorescent antibody techniques. Cheiron 11: 323-25.

Reddy, K.B (2012) Clinico-epidemiological studies on canine parvoviral infection in and around Tirupati.M.V.Sc thesis submitted to Sri Venkateswara Veterinary University,Tirupati.

Reddy, K.B., B.Shobhamani , B.Sreedevi,D. Rani Prameela,B.S.Reddy (2015) Diagnosis of Canine Parvo Viral infection in Dogs by Using Haemagglutination and Haemagglutination Inhibition Tests. Int. J .Vety .Health. Sci. Res. 3(3): 46-48.

Sagar, K.A (2005) Parvo viral infection in canine: Prevalance, diagnosis and therapy. M.V.Sc. thesis submitted to Indira Gandhi Krishi Viswa Vidyalaya, Raipur (C.G).

Sakulwira, K., K.Oraveerkul and Y. Poovorawan (2001) Detection and genotyping of canine parvovirus in enteric dogs by PCR and RFLP. Science Asia.27: 143-147.

Sanjukta, R., Mahesh Kumar, R.Mandakini (2011) Epidemiological study of canine parvovirus. Ind. Vety. J. 88 (12): 77-99.

Savi, J., P. Minakshi and G. Prasad (2009) Genotyping of field strains of canine parvovirus in Haryana using PCR and RFLP. Ind. J. Ani. Sci.79(10):971-97.

Savi, M., Meenakshi and G.Prasad (2009) Cloning and sequencing of canine parvovirus vaccine strain and field isolates from Haryana. Paper presented at XIIth ISVIB Conference held at CCSHAU, Hisar, 8-10 February 2009.

Schmitz, S., C.Coenen, M.Konig ,Heinz-Jurgen Thiel,R. Neiger (2009) Comparison of three rapid commercial canine parvovirus antigen detection tests with electron microscopy and Polymerase chain reaction. J. Vety. Diagn. Invest. 21: 344-345.

Schultz, R (2006) Duration of immunity for canine and feline vaccines: A review. Vety.Microbiol. 117(1):75-79.

Senda, M., N.Hirayama,H.Yamamoto and K.Kurata (1986) An improved haemagglutination test for study of canine parvovirus. Vety.Microbiol. 12:1-6.

Shackelton, L A., C.R. Parrish, U.Truyen and E.C.Holmes (2005) High rate of viral evolution associated with the emergence of carnivore parvovirus. Proceedings of the National academy of Sciences, USA. 102:379-84.

Shashidhara Y. M. and S. Kapil (2009) Simple tests for rapid detection of Canine parvovirus antigen and Canine parvovirus-specific antibodies. Clinical Vaccine Immunology. 16(1): 127-131.

Shima, F. K., T.T.Apaa, J.I.T.Mosugu (2015) Epidemiology of Canine Parvovirus Enteritis among Hospitalized Dogs in Effurun/Warri Metropolitan Region of Delta State, Nigeria. Open Access Library J. 2(1):1–7.

Silva, M.M.O., T.XCastro,E.M Costa,T.A.L.Trancoso,F.Mendes-De-Almeida,N.V.Labarthe And R.C.N.Cubel Garcia,(2013) Comparison of three laboratorial tests for diagnosis of canine parvovirus infection. Arq.Bras. Med. Vet. Zootec. 65:134-136.

Singh, D., A. K. Verma, and A.Kumar (2014) Phylogenic analysis of canine parvovirus isolates from Mathura, India. Veterinariski Archiv. 84(5): 505-512.

Singh, D., A.K.Verma ,A. Kumar, M.K.Srivastava, S.K.Singh, A.K.Tripathi,A.Srivastava and I.Ahmed (2013). Detection of canine parvovirus by polymerase chain reaction assay and its prevalence in dogs in and around Mathura, Uttar Pradesh, India.Ameri. J. Biochemistry and Molecular Biology. 3: 264-70.

Srinivas, V. M. V.,H.K.Mukhopadhyay, J.Thanislass , P.X.Antony and R.M.Pillai (2013) Molecular epidemiology of Canine Parvovirus in southern India. Vety.World. 16:744-49.

Srinivasan (2014) Canine parvovirus isolates of India and the relevance of Canine Parvovirus type-2 vaccines. J. Adv. Vet. Res. 4(1):34-41.

Stann S E, R.F.Digiacomo, W.E.Giddens and J.F. Evermann (1984) Clinical and pathological features of parvoviral diarrhoea in pound-source dogs. J. Ameri. Vety.Medical Asso. 185:651-55.

Steinel, A., E.H.Venter , M.Van Vuuren , C.R.Parrish and U.Truyen (1998) Antigenic and genetic analysis of canine parvoviruses in southern Africa. Onderstepoort J.Vety.Res. 65(4): 239-42.

Strassheim, M. L., A.Gruenberg, P.Veijalainen, J.Y.Sgro and C.R.Parrish (1994) Two dominant neutralizing antigenic determinants of canine parvovirus are found on the threefold spike of the virus capsid. Virology. 198:175-84.

Subhashini, C. R.,S. Meelarani, P.Ramadass and K.Nachimuthu (1997) Polymerase chain reaction and latex agglutination test for detection of canine parvovirus infection. Ind. J. Virol. 13(1):65-68.

Sundaran, S.T.,R.Ambily,R.S.Nair and M.Mini(2015) Utility of a rapid immunochromatographic strip test in detecting canine parvovirus infection compared with polymerase chain reaction. Vet. World. 8: 523-526.

Tattersall P and S.F.Cotmore (1990) Handbook of parvovirus.1st edition (Ed. Tijssen P) 12340.

Thomas, J.,M.Singh, T.K.Goswami, S. Verma and S. K.Badasara (2014) Polymerase chain reaction epidemiological investigation of canine parvoviral disease in dogs at Bareilly region. Vety.World. 7(11): 929-932.

Thomas, S. J., M. Singh, T. K. Goswami, S. Verma (2017) Phylogenetic analysis of the partial VP2 gene of canine parvovirus-2 from the northern region of India. Vet. Arhiv. 87: 57-66.

Tinky S, R.Ambily , M.Mini (2015) Utility of a rapid immune chromatographic strip test in detecting canine parvovirus infection compared with polymerase chain reaction. Vety.World. 8 (4): 523-526.

Truyen, U., J.F.Evermann, E.Vieler and C.R.Parrish (1996) Evolution of canine parvovirus involved loss and gain of feline host range. Virology. 215:186-89.

Tsao,J., M.S.Chapman,M. Agbandje, W.Keller, K.Smith, H.Wu, M.Luo, T.J.Smith, M.G.Rossmann , R.W.Compans and C.R.Parrish (1991) The three-dimensional structure of canine parvovirus and its functional implications. Science.251:1456- 64.

Umar, S., A.Ali, A.,M.Younus,M.K.Maan,S.Ali, W.A.Khan, W.A., M.Irfan (2015) Prevalence of Canine Parvovirus Infection at Different Pet Clinics in Lahore, Pakistan. Pakistan J. Zoology.l47: 657-663.

Vakili, N, B.Mosallanejad, R.Avizeh, S.Shapouri, M.Pourmahdi (2014) A comparison between PCR and Immunochromatography assay (ICA) in diagnosis of hemorrhagic gastroenteritis caused by Canine parvovirus. Arch. Razi Inst. 69 (1): 27-33.

Vieira,F.V., D.J.Hoffmann,C.U.F.Fabri (2017) Circulation of canine parvovirus among dogs living in human-wildlife interface in the Atlantic forest biome, Brazil. Heliyon.3(12):e00491.

Weichert, W. S., J.S.Parker, A.T.M.Wahid,S.F.Chang, E. Meier and C.R.Parrish (1998) Assaying for structural variation in the parvovirus capsid and its role in infection. Virology. 250:106-17.

Yang, Dong-Kun., Kyung-Suk Kang, Hyun-Ye Jo, Ha-Hyun Kim, Sung-Suk Choi, Jae-Young Song (2015) Isolation and identification of canine parvovirus type 2b in Korean dogs. Korean J Vet Res (2015) 55(3) : 163~167.

Yule, T. D., M.B.Roth, K.Dreier, A.F. Johnson, M.Palmar-Densmore, K.Simmons and R.Fanton (1997) Canine parvovirus vaccine elicits protection from the inflammatory and clinical consequences of the disease. Vaccine. 15:720-22.

Zhao H. (2015) Typing of Canine Parvovirus Strains Circulating in NorthEast China. Trans. and Emer. Dis.64: 495–503.

Zhao Y., Y.Lin, X.Zeng, C.Lu and J.Hou (2013) Genotyping and pathobiologic characterization of canine parvovirus circulating in Nanjing, China. Virol. J.10:272-372.

APPENDICES

A) Reagents used for sample processing

10% (W/V) phosphate buffered saline (PBS, pH 7.2)

Phosphate buffered saline (PBS) pH 7.2

Sodium chloride (NaCl)	: 8.0 g
Potassium chloride (KCl)	: 0.20 g
Disodium hydrogen phosphate (Na2HPO4.2H2O)	: 1.44 g
Potassium dihydrogen phosphate (KH2PO4)	: 0.24 g
TGDW	: 800 ml

Dissolve and adjust pH to 7.2 with HCl. Add TGDW to 1000 ml. Sterilize by autoclaving

B)Reagent used for HA test.

Alsever's solution -

D-Glucose	-2.05 gm
Citric acid	-0.055 gm
Sodium citrate	-0.8 gm
Sodium chloride	- 0.42 gm
Distilled water	-100 ml

Mix it well and sterilize in autoclave at 1160C for 10 min.allow to cool and stored in the refrigerator.

C)Reagents/buffers for polymerase chain reaction

i) Agarose

Agarose (Invitrogen) was used for the preparation of Agarose gel.

Agarose	: 2.0 gm
TAE buffer	: 100 ml

Heat the suspension to completely dissolve the agarose.

ii) Ethidium bromide (10 mg/ml)

Ethidium bromide	: 1 g
TGDW to make	: 100 ml

Stir on magnetic stirrer for several hours and store in dark bottles at room temperature.

iii) Gel loading buffer (6X)

Bromophenol blue	: 0.25 g
Sucrose	: 40 g
TGDW to make	: 100 ml

iii) TAE buffer (50X)

Tris base	: 12.1 g
Glacial acetic acid	: 2.85 ml
0.5 M EDTA	: 5 ml
TGDW to make	: 50 ml

D) Reagents for Isolation of Canine parvovirus-2

i) Fetal Bovine Serum

Fetal bovine serum (FBS) procured from Hi-Media (India) was used in this study.

ii) Antibiotic and Antimycotic solution

Antibiotic and antifungal solution (100X) containing streptomycin (10 mg/ml), penicillin (10,000 units) and amphotericin B (25µg/ml) from Sigma (USA) was used in the study.

iii) L-glutamine

The L-glutamine used in media preparation was procured from Amresco (USA). A solution of 30mg/100ml of L-glutamine was used in the study.

iv) Growth and Maintenance Media

DMEM (Dulbecco's Modified Eagle's Medium) (Sigma, USA) with Sodium bicarbonate and L-glutamine was used for propagation and maintenance of MDCK cell line and for cultivation of Canine parvovirus.

v) Growth medium (GM)

Stock DMEM : 89ml
Foetal Bovine Serum : 10ml
L-glutamine (30mg/100ml) :1.0ml
Antibiotic and antimycotic (100X) :0.1ml

The pH was adjusted to 7.2. The growth medium was filtered through 0.22µm membrane filter (47mm diameter) (Millipore, USA) and after sterility checking for 72 hours at 37°C, the medium was kept at 4°C until further use.

vi)Maintenance medium (MM)

The composition of the maintenance medium was similar to growth medium except that the fetal bovine serum was added at the rate of 1 per cent instead of 10 per cent.

Stock DMEM : 89ml
Fetal Bovine Serum : 1.0ml
L-glutamine (30mg/100ml) :1.0ml
Antibiotic and antimycotic (100X) : 0.1ml

The pH was adjusted to 7.2. The maintenance medium was filtered through 0.22µm membrane filter (47mm diameter) (Millipore, USA) and after sterility checking for 72 hours at 37°C, the medium was kept at 4°C until.

vii) Trypsin Phosphate Glucose Versine (TPGV) solution

Trypsin : 100mg
Versine (EDTA) : 200mg
Glucose : 500mg
Phosphate buffered saline : 100ml

It was subjected to filtration with 0.22µm membrane filter (Millipore, USA) and was stored in aliquots at 4°C.

VITA

The author, **Ku.Payal Rajesh Dorlikar** born on 7th July 1994 at Bhandara (M.S). She completed her S.S.C. Examination in year 2009 from Nutan Kanya school,Bhandara. In first division with distinction and H.S.S.C. Examination in year 2011 from Nutan Kanya Junior College, Bhandara, in first class.The author has successfully completed her B.V.Sc & A.H. degree course in year 2016 from Nagpur Veterinary College, Nagpur. She was active member of sports academy.

For further studies in the year 2016, she joined M.V.Sc.in the subject of Veterinary Microbiology in Nagpur Veterinary College, Nagpur.The author attended IAVMI 2017 and 2018 conference held on Maharashtra and Tirupati,Andhra Pradesh and presented research paper during her post graduation.

THESIS ABSTRACT

a)	Title of thesis	:	**MOLECULAR CHARCTERIZATION OF CANINEPARVOVIRUS-2 INFECTION IN AND AROUND NAGPUR**
b)	Full Name of Student	:	**DORLIKAR PAYAL RAJESH**
c)	Name & Address of Advisor/ Guide	:	**Dr.S.R.WARKE** **Assistant Professor** **Department of Veterinary Microbiology and Animal Biotechnology, T & R Cell,** **Nagpur Veterinary College, Nagpur**
d)	Degree to be awarded	:	**MASTER OF VETERINARY SCIENCE**
e)	Year of award of degree	:	**2018**
f)	Major subject	:	**VETERINARY MICROBIOLOGY**
g)	Total number of pages in the thesis	:	**60**
h)	Number of words in the thesis abstract	:	**636**
i)	Signature of student	:	
j)	Signature, Name & Address of forwarding authority	:	**Associate Dean** Nagpur Veterinary College, Nagpur

---

ABSTRACT

Canine parvovirus is an important pathogen of dogs and is responsible for serious occurrences of morbidity and mortality, in spite the availability of safe and successful vaccines. The virus belongs to the family *Parvoviridae* .Canine parvovirus causes hemorrhagic enteritis and bloody diarrhea in puppies above the age of 12 weeks.CPV emerged as a new virus in the late 1970s.Now, the

disease condition has been complex further due to development of new antigenic variants namely CPV-2a, CPV-2b, and CPV-2c above the years.VP2 is the major capsid protein of CPV which responsible for the evolution of diverse antigenic types. The virus is widely prevalent throughout the world and a more number of incidences have been reported from India by various workers. The present study was undertaken to study the incidence of Parvoviruses among canines in and around Nagpur using conventional methods and molecular tools, further an isolate were subjected for the phylogenetic relationship of Parvoviruses based on VP2 gene sequence of field isolate.

In the present study, total 91 faecal samples/rectal swabs collected from dogs suspected for CPV-2 and screened for CPV antigen by HA, Ubio quickVET kit and PCR. The incidence of CPV by these tests was found to be 23.91%, 35.86% and 45.05% respectively. Age wise distribution showed that maximum positive samples belong to age group of 0-3 (26.66%) followed by 4-6 (23.02%) and then 6-9 (14.28%). Highest prevalence was observed in male dog (30.18 %). Breed wise examination of data indicated that highest prevalence in German shepherd (36.36%), followed by Pomerian (30 %), Labrador (28.71%) Great Dane (27.77%), Doberman (18.75%), andNon descript (17.39%).Three samples were positive with prevalence of 17.8% in vaccinated and 30.15 % in non vaccinated dogs by HA.

Age wise distribution showed that maximum positive samples belong to age group of 0-3 (51.11 %) followed by 4-6 (25.64%) and then 6-9 (14.28%). Highest prevalence was observed in male dog (43.39%).Breed wise analysis of data indicated that highest prevalence in German shepherd (72.72%), followed by Pomerian (40 %), Doberman (25%).Four samples were positive with prevalence of 14.28 % in vaccinated and 46.03 % in non vaccinated dogs by Antigen detection kit.

Age wise distribution showed that maximum positive samples belong to age group of 0-3 (57.77%) followed by 4-6 (35.89%) and then 6-9 (14.28%).Highest prevalence was observed in male dog (49.05%) than female (39.46%).Breed wise analysis of data indicated that highest prevalence in German shepherd (72.72%), followed by Pomerian (50%), Doberman (43.75%).8 samples were positive with prevalence of 28.57% in vaccinated and 52.38 % in non vaccinated dogs by PCR.Indicating PCR exist more sensitive and specific.

In the present study, sensitivity and specificity of antigen detection kit (Ubio quickVET kit) compared to PCR was found to be 78.08%and 96% respectively. PCR detected more number of positive samples than Antigen detecting kit indicating that PCR is more sensitive than Antigen detection Kit.

For the sequence analysis, a sample randomly selected on the basis of amplicon of PCR, the sequence analysis revealed that the samples were CPV-2b antigenic type. Sequence was submitted to the genebank and was assigned accession number (LC404128) for CPV-37. Phylogenetic analysis revealed has 53 and 54% homology with the Argentina of Canine Parvovirus sequence. The sequence in the present study formed a separate node from rest of the Indian isolates indicating regional variation in the CPV isolates. The finding suggested that more survey needed to know the true prevalence and significance of CPV worldwide.

A PCR positive sample was adopted to grow in MDCK cells and produced typical cytopathic effect with normal architecture of cells got disrupted and rounding of cells occurs along with clumping and finally detachment of cells has been marked after 96 hours. The cell culture fluid was collected to extract DNA that was subjected to PCR. CPE positive samples amplified 681 bp product in PCR.

प्रबंध सारांश

अ.	प्रबंधाचे शिर्षक	:	**श्वानांनामधील पाइव्हो विषाणु—२ रोगाचे नागपुर आणि सभोवतातील विभागातुन सुक्ष्म रेणविय गुणवर्णन**
ब.	विद्यार्थ्याचे पुर्ण नांव	:	**डोरलीकर पायल राजेश**
क.	मार्गदर्शकाचे नांव व पत्ता	:	**डॉ. शुभांगी रा. वारके** **सहाय्यक** प्राध्यापक पशुवैद्यकिय सुक्ष्मजिवशास्त्र व पशुजैवतंत्रज्ञान विभाग, नागपूर पशुवैद्यक महाविद्यालय, नागपूर
ड.	प्रदान करण्यात येणारी पदवी	:	**स्नातकोत्तर पदवी (एम.व्ही.एस.सी.)**
इ.	पदवी प्रदान करण्याचे वर्ष	:	**२०१८**
फ.	मुख्य विषय	:	**पशुवैद्यकिय सुक्ष्मजिवशास्त्र**
ग.	प्रबंधातील एकूण पृष्ठ	:	**६०**
ह.	सारांशातील एकूण शब्द	:	**५३३**
ई.	विद्यार्थ्याची सही	:	
ज.	अग्रेषित करणाऱ्या अधिकाऱ्याची सही, नांव आणि पत्ता	:	

सहयोगी अधिष्ठाता
नागपूर पशुवैद्यक महाविद्यालय, नागपूर

सारांश

श्वानांचा पार्व्होव्हायरस हा पार्व्होव्हीरीडे कुटुंबातील असूण हा श्वानानांमधील महत्वाचा रोग आहे. याविषाणुकरीता सुरक्षित आणि प्रभावी लस

उपलब्ध असून सुद्धा रोगप्रादुर्भाव व मृत्युदर आढळुन येतो. श्वानांनच्या पार्व्होव्हायरस मुळे १२ आठवडयांपेक्षा अधिक वयांच्या पिल्लांनमध्ये आतडयांचा रक्तस्त्रावासोबत दाह असतो आणि रक्ताचा अतिसार होतो. १९७० च्या दशकामध्ये सी.पी.व्ही. हा विषाणू उदयास आला. आतापर्यंतच्या वर्षभरात मिश्र स्वरूपात मुख्य:त विविध ॲंटिजनिक असलेले सी.पी.व्ही.—२ए, एक्स.पी.व्ही.—२बी आणि सी.पी.व्ही—२सी यामुळे रोगस्थिती तयार झालेली आहे. व्ही.पी.—२ हा सी.पी.व्ही. विषाणुचा बाहय प्रथिने असूण विविध ॲन्टीजनिक प्रकार उत्पन्न करण्याकरिता जबाबदार आहे. हा विषाणू संपूर्ण जगभरात प्रचलित असूण हया विषाणूचा प्रार्दुभाव जास्त प्रमाणात भारतामध्ये काम करण्याऱ्या लोकांकडून नोंदवण्यात आला. सदर अभ्यास क्रमात पारंपारिक पद्धती आणि सुक्ष्म रेणबिय गुणवर्णनांचा वापर करून श्वानांनमधील पार्व्होव्हारस चा नागपूर व सभोवतातील भागांचा वंशावळीक विश्लेषणानुसार व्ही.पी.—२ जनुक विश्लेषण विष्टेच्या नमुण्यांमधून विविध क्षेत्रातुन करण्यात आले.

सदर अभ्यास क्रमात सी.पी.व्ही.—२ विषाणू करता, संशयित असलेल्या एकुण ९१ विष्टेचे/ गुद्द्वारेचे नमुने घेण्यात आले आणि सी.पी.व्ही. विषाणूंची प्रतिजन चाचणी एच.ए., युबीको क्विक व्हेट किट आणि जनुकीय शृंखलेद्वारे छाणणी करण्यात आली. सी.पी.व्ही विषाणूचा प्रघात याच चाचण्याणद्वारे २३.९१ टक्के, ३५.८६ टक्के आणि ४५.०५ टक्के इतका आढळला. सी.पी.व्ही. विषाणुंचा सर्वात जास्त प्रादुर्भाव हा नर श्वानांनमध्ये एच.ए. प्रतिवजन चाचणी किट, जनुके शृंखलाद्वारे आढळला. वयानुसार पार्व्होव्हारस विषाणुचे वितरण केले असता सर्वात जास्त सकारात्मक नमुणे ०—३ वयोगटातील (२६.६६ टक्के) ४—६ वयोगटातील (२३.०२ टक्के) आणि ६—९ वयोगटातील (१४.२८ टक्के), एच.ए. चाचणी द्वारे करण्यात आले. तसेच प्रतिजन चाचणी किट द्वारे ०—३ वयोगटात (५१.११ टक्के) ४—६ वयोगटात (२५.६४ टक्के) तर ६—९ वयोगटात (१४.२८ टक्के) असे

आढळले. तसेच जनुके शृंखलेद्वारे ०—३ वयोगटात (५७.७७ टक्के), ४—६ वयोटात (३५.८९ टक्के) तर ६—९ मध्ये (१४.२८ टक्के) आढळतो. जातीनिवाह वर्गिकरणानुसार सर्वात जास्त सकारात्मक नमुने एच.ए. चाचणीद्वारे जर्मन शेर्पड (२६.३६ टक्के) पोमेरियन (३० टक्के), लॅब्राडोर (२८.७१ टक्के) ग्रेट डेन (२७.७७ टक्के) डोर्बरमान (१८.७५ टक्के) व नॉन—डिसक्रीप्ट (१७.३९ टक्के) आढळले. तसेच लसीकरण केलेल्या ३ नमुण्यामध्ये (१७.००८ टक्के) आणि लसीकरण न केलेल्या (३०.१५ टक्के) असा प्रघात आढळला. तसेच प्रतिजन चाचणी किट द्वारे जर्मन शेपर्ड (७२.२२ टक्के) पोमेरियन (४० टक्के) लॅब्राडोर (२५ टक्के) असा आढळले. ४ लसीकरण केलेल्या नमुन्यांमध्ये (१४.२८ टक्के) तर लसीकरण न केलेल्या श्वानांनमध्ये (४६.०३ टक्के) प्रघात आढळला. तसेच जनुके शृंखला प्रतिक्रीयेद्वारे जर्मण शेर्पड (७२.७२ टक्के) पोमेरियन (५० टक्के), डोर्बरमान (४३. ७५ टक्के) असा आढळुण आला. ८ लसीकरण केलेल्या (२८.५७ टक्के) आणि लसीकरण न केलेल्या श्वानांमध्ये ५२.३८ टक्के नमुन्यांनमध्ये सकारात्मक प्रघात आढळला. त्यामुळे असे आढळले की जनुके शृंखला प्रतिक्रीया अधिक संवेदनशील आणि विशिष्ट आहे. सदर अभ्यासात जनुके शृंखलेच्या तुलनेत प्रतिजन चाचणी किट ची संवेदनशीलता आणि विशिष्टता ७८.०८ टक्के आणि ९६ टक्के आढळली.

क्रम विश्लेषणानुसार सरसकट निवडलेले जे नमुणे जनुके शृंखलेद्वारे सकारात्मक होते ते नमुणे सी.पी.व्ही.—२बी प्रतिजनीक प्रकारचे आढळले. सी.पी. व्ही—३७ करीता क्रम जनुकेय बँकेत जमा केले असता त्याला एल.सी. ४०४१२८ असा सांकेतिक क्रम देण्यात आला. वंशावळी विश्लेषणानुसार श्वानांनमधील पार्व्होव्हासरस विषाणू ५३ टक्के आणि ५४ टक्के अरजेंटीना सोबत अधिक साम्यता दर्शवितो. सदर अभ्यासातील क्रम भारतातील इतर नमुण्यांनसोबत एक वेगळा नोड तयार केला असूण सी.पी.व्ही विषाणुंची नमुण्यामध्ये विभागिय विविधता आढळते.

सदर शोधाद्वारे असे लक्षात येते की संपूर्ण देशात सी.पी.व्ही. विषाणुचा खरा प्रादुर्भाव आणि महत्व जाणून घेण्याकरिता अधिक सर्वेक्षण करण्याची गरज आहे.

एम.डी.सी.के. पेशींद्वारे जनुके शृंखलेमध्ये सकारात्मक नमुण्याची तपासणी केली असता संपूर्ण पेशींचा आकार विस्कळीत झाला आणि पेशी गोलाकार होऊन एका ठिकाणी गोळा झाल्या तसेच ९६ तासांनंतर पेशी पूर्णत: विस्कळीत होऊन पेशीचे विभाजन झाले असे विशेष सॉटोपाथीक परिणाम आढळले. सी.पी.व्ही. विषाणूची एम.डी.सी.के. पेशींमध्ये ठराविक सॉटोपाथीक परिणाम दर्शविलेल्या पेशीच्या द्रवातुन ६८१ बी.पी. जनुके असल्याचे जणुकेय शृंखलेद्वारे पृष्टी करण्यात आली.