# Molecular detection of canine distemper virus in clinically suspected dogs

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**VETERINARY EPIDEMIOLOGY AND PREVENTIVE MEDICINE**

**By**

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# CERTIFICATE-I

This is to certify that the thesis entitled **"Molecular detection of canine distemper virus in clinically suspected dogs"** submitted in partial fulfilment of the requirements for the award of the degree of **Master of Veterinary Science (Veterinary Epidemiology and Preventive Medicine)** to the Odisha University of Agriculture and Technology is a faithful record of bonafide and original research work carried out by **Ashok Kumar Dash**, **Adm. No. 18192J04** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

It is further certified that the assistance and help received by him from various sources during the course of investigation has been duly acknowledged.

**CHAIRMAN**
**ADVISORY COMMITTEE**

# CERTIFICATE-II

This is to certify that the thesis entitled **"Molecular detection of canine distemper virus in clinically suspected dogs"** submitted by **Ashok Kumar Dash Adm. No. 18192J04** to the Odisha University of Agriculture and Technology, Bhubaneswar in partial fulfilment of the requirements for the degree of **Master of Veterinary Science (Veterinary Epidemiology and Preventive Medicine)** has been approved/disapproved by the students' advisory committee and the external examiner.

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**Place: Bhubaneswar**

**Date:** **(Ashok Kumar Dash)**

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# ABBREVIATIONS

| | | |
|---|---|---|
| % | : | Per cent |
| & | : | And |
| µl | : | Microleter |
| CD | : | Canine Distemper |
| CDV | : | Canine Distemper Virus |
| Conc. | : | Concentration |
| Dl | : | Decilitres |
| Fig. | : | Figure |
| g | : | Gram |
| Hb | : | Haemoglobin |
| Hrs | : | Hours |
| i.e. | : | That is |
| IU | : | International Unit |
| ml | : | Millilitres |
| mm | : | Millimetres |
| ng | : | Nanogram |
| PCR | : | Polymerase Chain Reaction |
| PCV | : | Packed Cell Volume |
| TEC | : | Total Erythrocyte Count |
| TLC | : | Total Leucocyte Count |

# ABSTRACT

Canine distemper is extremely infectious vaccine preventable viral disease. It caused by canine distemper virus (CDV) that is coming under morbillivirus. CDV is a ssRNA virus. A study entitled, 'Molecular detection of canine distemper virus in clinically suspected dogs' was chalked out with the objective to detect CDV in domestic pups clinically suspected for distemper. Cases presented either in the Teaching Veterinary Clinical Complex or peripheral Government veterinary dispensary of Bhubaneswar city during the period June 2019 to July 2020 had the clinical signs of biphasic fever, profuse salivation, mucopurulent ocular discharge, chorea, posterior weakness and convulsion. Conventional polymerase chain reaction was performed using designed primer i.e., 1622 (F) - (ACCTGGGACCAGTGAAGAGA) HPSF 0.01 scale, 1864 (R)- (TCTCTCTGAGGGCTTTGAGG) HPSF 0.01 scale) that targeted N gene of CDV. Amplification was recorded with amplicon size of 243 bp in samples collected from four pups. Sequencing followed by BLAST results showed 97.13 per cent homology with canine distemper virus. In this study haematological alterations were seen in CD positive pups by auto haematological analyzer. Alterations in haematological parameters were recorded with respect to haemoglobin and total leucocyte count. Molecular diagnosis of CDV through cPCR from pets is first of its kind in this region that confirmed circulation of such virus in the unvaccinated susceptible hosts. The study helped in early and accurate detection of CDV.

# INTRODUCTION

Canine distemper (CD) is a vaccine preventable viral disease and its fatality rate is regarded as second to that of rabies. The etiological agent, canine distemper virus (CDV), belongs to the genus *Morbillivirus* in the family *Paramyxoviridae*. CDV causes infection in a broad range of animals namely Canide (dog, fox, coyote, dingo, wolf, and jackal); Mustelidae (ferret, mink, martens, skunk, badger, otter); Procyonidae (olingos, ringtails); Felidae (Tiger, cheetah, lion, cougar, jaguar, margay, ocelot); Ailuridae (red panda); Hyaenidae (hyena); Ursunidae (polar bear) and Viverridae (African civet). It has consequently been acknowledged in some non-human primates, posing a conservation threat to several free-ranging and captive non-domestic carnivores (Beineke *et al*., 2015 and Deem *et al*., 2000). Infected carnivore reveals multi-systemic signs affecting the gastro-intestinal, respiratory, muscular and neurological systems (Namroodi *et al*., 2013 and Sykes, 2014).

Canine distemper virus is a single-stranded, non-segmented RNA virus. CDV is negative sense virus with diameter of about 100 to 250 nm (Murphy *et al*., 1999). Its genome size is 16,000 Kb with encoding one non-structural protein (C) and six structural proteins (Lamb and Parks, 2014). Only one serotype of CDV is present but its strains differ in virulence. Several relative studies have revealed that the H gene is put forwarded to a greater genetic unevenness than the other CDV genes, which makes it appropriate for genetic study (Haas *et al*., 1999).

Mostly, CDV affects vulnerable animals by inhalation. Following aerosol infection the virus replicates in the mast cells, macrophages and lymphocytic cells of the upper respiratory tract. Systemic dissemination of the virus is mediated by infected cells, such as lymphocytes, monocytes, platelets. Propagation of virus occurs through non-cell-associated virus. It leads to infection of various organs. The commencement of the disease is accompanied by gastrointestinal, respiratory, integumentary and nervous signs. The extent and harshness of the disease depends mainly on the immunity of the CD affected animals. The extent of infectivity depends on how quickly an animal adopting an immune response to CDV. However, if immune response is below threshold level then the virus propagates to various tissues causing an acute or chronic disease with huge fatality (Latha *et al*., 2007b). The

infection advances to central nervous system (CNS) and may cause mortality (Riley and Wilkes, 2015).

Canine distemper virus is a pantropic virus. It causes immunosuppression. However, many dogs do not exhibit classical clinical presentation and infected with other pathogens that are responsible for similar signs, rendering CD diagnosis difficult. Canine distemper is sometimes confused with other systemic infectious diseases like canine leptospira, infectious canine hepatitis (ICH), Rocky Mountain spotted fever. CDV are shed frequently in the ocular and nasal secretions (Eghafona *et al*., 2007 and Gencay *et al.,* 2004). Routine laboratorial tests may provide some useful clinical information which can be used in diagnosis of the CD. Decrease in lymphocyte count and neutrophil count in circulation with elevation of liver and muscle enzymes (AST, ALT, CPK, BUN and creatinine) were steady findings of CD.

Various samples including conjunctiva swabs, nasal swabs, blood smears and have been used for the diagnosis of the disease (Alldinger *et al*. 1993 and Sishu *et al.*, 1993).The virus produces inclusions bodies. Inclusion bodies are of variable shapes and sizes which are present in different cells and can be made for use for diagnosis of the disease (Alleman *et al*., 1992 and Kapil *et al*., 2008). However, many tests like virus neutralization test, Immunohistochemistry, indirect immunofluorescence, hematology, immunochromatographic assay, complement fixation test, ELISA, viral isolation and PCR assays aid in the rapid and confirmatory diagnosis of CDV. But PCR based diagnosis remains gold standard test. CDV has been most easily and efficiently detected in PCR assay from clinical specimens (Seki *et al.*, 2003).

Sequence scrutiny of CDV strains from various geographical regions and from various animal species has revealed that the N gene undergoes genetic drift and genetic shift according to geographical location (Martella *et al*., 2007). The hemagglutinin (H) and Neuraminidase (N) proteins are the most important antigens for inducing protective immunity against CD. Any variability of recent CDV strains may cause in vaccination failure (Li *et al*., 2014).

Seroprevalence of CDV had been studied in 70 % area from South India (Latha *et al*., 2007a). Research work on molecular epidemiology is lacking in many parts of India including Odisha. CDV vaccines are commercially available. CDV

vaccine is given in combination with other vaccines such as canine leptospira vaccine, infectious canine hepatitis vaccine, para-influenza vaccine and parvovirus vaccines. Recently usual vaccination against CD is almost regular to prevent the prevalence of the disease. The disease remains as a major threat to canine population even after regular vaccination (Temilede *et al.*, 2015). Regular periodical vaccines failures of CD are attributed to the circulating form of new virual strains that are genetically divergent to that the vaccine strains.

CD virus is a RNA virus. It is labile to extreme environmental conditions like heat, sunlight etc thereby making its isolation/detection from clinical samples difficult. In the view of the above facts, the present research work was proposed with the following objectives:

1. To detect canine distemper virus from biosamples in clinical cases.
2. To record the clinical and hematological alterations in clinical cases.

# REVIEW OF LITERATURE

## 2.1 The virus

Canine distemper (CD) was first explained by Henri Carre in 1905, is a highly infectious, communicable, incurable, highly fatal, viral disease that affects the integumentary, respiratory, CNS, muscular and gastrointestinal involving many systems (Allwin *et al*., 2016).

A huge number of canine populations, minks, foxes, ferrets die from CD every year, causing huge economic losses (Loffler *et al*., 1997) with high mortality rate in dogs after rabies.

The disease is characterized by high morbidity rate. The disease is also having high mortality rate in a broad-spectrum of protective-native hosts. The disease includes few non-human primates and also includes various vulnerable carnivores and wild carnivores (Beineke *et al*., 2015 and Martinez-Gutierrez *et al*., 2016)

## 2.2 Historical perspective

Canine distemper was probably seen as highly infectious and contagious disease of dogs since 1746 in Peru. McBridge (1870) in UK reported CD as the curse to the canine population since it attacked canines of every breeds and ages

A term of hard pad is coined by Rubarth in 1947, which varied from conformist CD due to development of hyperkeratosis of foot pads with frequent manifestations of encephalitis.

Gledhill in 1953 said *Haemophillus bronchisepticus* as the causative agent of CD in live dogs and post mortemed dogs which are showing respiratory signs and symptoms. He proposed CD as an acute, contagious, febrile disease with an incubation period of four days that was characterized by chorea, coryza, conjunctivitis, biphasic fever, severe gastrointestinal changes and discrete symptoms but with infrequent observation of neurological expressions of disease.

Dunin and Laidlaw *et al*. (1926) described that the disease in ferrets was communicable to dogs and vice- versa.

## 2.3 Distribution

### 2.3.1 Abroad

Calderon *et al.* (2007) found the CDV in both vaccinated and unvaccinated dogs in Argentina.

Simon- Martinez et *al.* (2008) detected the CD from serum samples by RT-PCR in Mexican dogs and found that there was difference in N gene sequences obtained from vaccine and the circulating strains and proposed that original CDV lineage might be present in Mexico.

Lan *et al.* (2009) found first isolation of American-1 genotype of CDV from Vietnamese dogs.

Van Nguyen *et al*. (2017) found H gene from five out of 43 suspected dogs in Vietnamese and successfully isolated two CDV H genes in a canine slam expressing cell lines.

Namroodi *et al*. (2013) found the CDV in rural dogs of Iran by conventional PCR and combination with Nested PCR and concluded the n-PCR is responsive and relevant approach for analysis of CDV.

Eha *et al*. (2014) detected and quantified the viral RNA by real time PCR in Italy.

Zhao *et al*. (2014) reported the emergence of CD strains with three amino acid changes over in the Haemagluttin protein of CDV from breeding minks, raccoon dogs and foxes having no history of vaccination record in North Eastern China.

Fischer *et al.* (2016) reported the CDV epidemics in South America. Out of 172 CDV suspected samples collected from Brazil revealed 89 positive for CDV, generating 43 new CDV sequences.

Pope *et al*. (2016) tested CDV in raccoons, gray foxes and revealed that CD comes into view to be widespread in Eastern Tennessee wildlife. They concluded that recovered shudders act as a possible source for CD outbreak in domestic dogs and the circulating genotype was genetically discrete from vaccine strain.

Loots *et al*. (2018) reported the CDV in South African Wildlife and indicating that CDV strains encoding 519I/519H were better tailored to non-canine species than canine species.

### 2.3.2 India

In India, CDV infections are relatively frequent. The disease is confirmed by serological survey. Here diagnostic technique used is ELISA (Parthiban *et al*., 2000; Ramdass and Latha, 2001).

Latha *et al.* (2007a) screened 160 conjunctiva samples by using dot ELISA and indirect immunefluorescent assay as a standard technique for the detection of CDV and 112 (70%) were found positive. Their epidemiological survey revealed that the illness is seen in both males and females. The illness is the most frequent in canines having history of no vaccination record. The canines are in between 1- 5 years of age.

Vanak *et al*. (2007) studied that the confirmation for current and past contact to CDV was found in 91 % of individuals. In an inspection, the disease is found in free ranging canine population of Great Indian Bustard Sanctuary, Maharashtra- India.

Ramanathan *et al*. (2007) reported that 49 out of 56 lioness (87.5 %) positive for CD in their serological investigation on captive Asiatic lioness from West region of country India. They also described that Canine distemper (CD) and feline Parvovirus (FPL) is a most important risk to captive Asiatic lioness.

Srivastav and Nigam *et al*. (2010) described that CD is a main menace to the Asiatic wild canine species in India.

Pawar *et al.* (2011) found that the CDV N gene from ocular and brain tissue by using RT-PCR. They used B95a cell lines for proliferation of virus.

Seshadri *et al.* (2015) reported H and F gene of two field isolates of CDV from a total of 15 samples. They differentiated the field isolates from vaccine strain using sequence scrutiny and found that the field isolates forms a sequence Clade in the phylogenetic tree from the vaccine strain.

Swati *et al.* (2016) found three CDV positive samples from a total of 50 CDV suspected cases and they successfully isolated CDV isolates in MDCK cell lines after lymphocyte culture.

Ashmi *et al.* (2017) found the CDV N gene by using Reverse Transcrpitase-PCR and conventional PCR. Out of 90 samples collected, 19 samples (21%) showed positive by RT-PCR and 21 samples (23 %) by conventional PCR.

Buragohain *et al.* (2018) screened a total of 167 clinical samples and 58 cases (34.73 %) were found positive by conventional PCR. They had also found occurrence of CD was more in young dogs of 0-6 months (57%) of age group, in winter season (48.81%), local non- descript dogs (43.10%), unvaccinated dogs (72.41%) and male (58.62%) pups.

**2.4 Host range**

Recent studies by Munson *et al*. (2004) and Thalwitzer *et al*. (2010) on the serological survey of captive cheetah and free ranging cheetah (*Acinonyx jubatus*) from Nambia showed that cheetahs could be affected by CDV (sero-positive) but alike to domestic cats did not reveal clinical signs.

CDV has recently stretched to nonhuman primates (Deem *et al*., 2000 and Sakai *et al*., 2013).

The capability of CDV to toggle hosts has increased concern about the annihilation threat it poses to various vulnerable wildlife species (Ripple *et al*., 2014 and Viana *et al*., 2015).

CDV outbreak in large felines such as lions, leopards, and tigers have defied the belief that feline group of animals are resistant to CDV (DiSabatino *et al.*, 2014 and Stanton *et al*., 2002).

**2.5 Prevalence and Epidemiology**

Moller *et al*. (1993) reported an increase in canine distemper in the city canine population of the continent Copenhagen. The majority of confirmed cases (64.58%) were recognized in growing dogs aged 2.5 years or less. The cases were found in dogs

from 9 weeks to 12 weeks of age. The greater parts of cases were screened among dogs having no vaccination history. The cases were also found in animals without vaccination. Various rigorous clinical cases were also screened in animals having proper vaccination record.

Kommonen *et al.* (1997) carried out a study on outbreak of canine distemper in dogs having updated history of vaccination in the continent of Finland. From total of 860 confirmed cases, 626 (73 per cent) were detected between 2 and 24 months of age. 487 of these had been having a vaccination record at least once and 351 (41 per cent) had a complete record of vaccination details.

Greene and Appel (1998) reported that though canines of all breeds are vulnerable to CDV infection, Dolichocephalic (Doberman, German shepherd) breeds have higher prevalence as relative to brachicephalic breeds.

Headley and Graca (2000) carried out epidemiological studies on 250 cases of canine distemper. They described that dolichocephalic (Doberman, German shepherd) breeds were more vulnerable than brachicephalic breeds. Although the accurate cause for mixed- breed dominance to CDV infection was unknown; mongrels may receive a lesser amount of interest, and are believed to be more apt to wander and contact CDV carrier canines than their pure-breed counterparts, thereby raising their chance of infection. Major disparity in vulnerability was not seen between both sexes.

Jozwik and Frymus (2002) reported that pups in the age group of 3 to 6 months were more vulnerable to CDV infection. They described that most (66%) of the affected dogs had never been vaccinated against distemper, whereas (22%) of infected dogs were vaccinated at least once.

Latha *et al.* (2007) carried out appraisal of canine distemper virus affection in dogs having with or without history of vaccination. Both the sexes were equally affected by CDV infection and cases were high in spitz, a dolichocephalic breed. It was found that dogs in the age group between 1-5 years were vulnerable to CDV infection. The average T1/2 of maternal antibodies had been computed and the age of 3-6 months was found to be the suitable time to vaccinate the pups, as at this age there would been tire loss of maternal antibodies, which would otherwise hinder with the production of vaccine antibodies.

Nelson and Couto (2009) reported that canine distemper was more frequent in young dogs of <7 months of age with noticeable severity.

Lal *et al*. (2010) carried out a study on incidence of canine distemper in dogs. Out of 52 dogs screened, only 10 dogs were detected positive for canine distemper. The overall prevalence of canine distemper amongst dogs was found to be 19.23 per cent. Age wise occurrence of canine distemper in dogs was the highest in pups up to 6 months of age (40%) followed by 12-18 months, above 36 months (20% each), 6-12 months and 24-30 months (10% each) of age.

Dongre *et al*. (2013) reported the incidence of canine distemper infection in and around Mhow region of Madhya Pradesh. A total of 100 dogs were screened for incidence of CD infection, out of which 9 were detected positive for canine distemper virus infection. Thus, indicating 9 per cent incidence of CD. Young dogs were more affected and contributed highest of all CDV detected cases. Males were more vulnerable for CDV infection than females. Non-descript dogs contributed to 5 out of 9 of all CDV detected cases.

Behera *et al.* (2014) conducted a study on ten dogs (7 crossbred, 2 Mongrels and 1 Doberman; 6 males and 4 females) of 2.5 to 7 months of age which were presented to the outpatient department of the college of veterinary science and animal husbandry; CAU, Mizoram with clinical signs and symptoms of canine distemper. There was no sex or breed predilection. The disease was seen throughout the year mostly due to lack of vaccination and partly due to suitable environment in this part of the country both in summer and winter.

### 2.6 Genotype

Only one serotype of CDV was screened with several co-circulating genotypes based on variation in the M proteins (Ke *et al*., 2015). The H gene assortment is useful to scrutinize the molecular epidemiology of the virus. CDV have been detected worldwide, majority of them following an environmental pattern of distribution.

### 2.7 Morphology

Canine distemper virus (CDV) has non-segmented RNA virus. This RNA virus is negative sense, single stranded. RNA virus is approximately 16kb. CDV has

RNA genome. RNA virus is an enveloped virus particle. RNA virus is 200 to 300 nm in diameter (Murphy *et al*., 1999). Canine distemper virus encodes seven viral proteins. These proteins are phosphor-protein (P), small protein (L), nucleo-capsid (N), matrix protein (M), haemagglutinin (H), fusion protein (F), and one non-structural protein (C) ( Von Messling *et al*., 2001).

The non-structural proteins (C) are made by a closed reading frame in the N gene (Lamb *et al.,* 2001). The host cell consequential lipid envelope is pointed with transmembrane. Haemagglutinin (H), fusion glycoprotein internally the envelope become stabled by the matrix protein (VonMessling *et al*., 2001).

**2.8 Physiochemical properties**

CDV is extremely susceptible to UV radiation, heat, aridness, oxidizing agents, detergents and lipid solvents (Kingsbury *et al*., 1978).

It could be damaged by temperature of $50^0$C to $60^0$C in 30 minutes and also by routine disinfection procedures (Greene and Appel, 1998).

Zee *et al*. (1999) reported that CDV could survive for weeks at near freezing temperature and the virus infectivity would be lost at pH 10.4 and below 4.4.

**2.9 Transmission and stability**

CD is highly contagious, infectious and the key mode of CDV transmission is through aerosol droplets of respiratory and ocular exudates containing virus. Trans-placental transmission has been seen basing on field observation (Krakowka *et al*., 1977).

Greene and Apple (1990) and Williams, (2001) studied that during acute phase of infection, other body fluids like all excretions and secretions (urines, faeces and skin) could also contain virus.

Greene and Apple (1990) and Apple (1987) reported that viral shedding might pursue infection for up to 90 days and could occur even if the animal was sub clinically infected.

Butler *et al*. (2004) studied that domestic dogs, from population covering safety wildlife regions, were often having history of no vaccination record. It occurs in high compactness with a rapid population turnover. These canines and wildlife come into get in touch with as both may ramble several kilometers in and out of the covered regions.

Pathogen upholding in the system was further augmented through intra-species spread of CDV in an extensive type of hosts (Alexander *et al*., 2010).

**2.10 Pathogenesis**

Incubation of CDV could fluctuate 1 to 4 weeks and could vary in disease staging i.e. from no clinical signs or subclinical infection in 30-80 % of dogs to neurologic signs and then death. Beineke *et al.* (2009).

Kapil *et al*. (2011) and Martella *et al*. (2008) studied that the first viremic phase, which consists of high viral duplication and systematic spreading throughout lymph tissues, results in immunosuppression and fever. A second viremic phase occurs 6-9 days after infection and consists of the comprehensive infection of parenchyma and epithelial cells all over the body. An additional obscuring feature is the growth of secondary bacterial or protozoan infections as an effect of immunosuppression.

Kapil *et al*. (2011) reported that neurological disease typically occurs 1-3 weeks following initially clinical signs and symptoms and has both acute and chronic phases.

**2.11 Clinical signs**

Development of a biphasic fever represents characteristics clinical findings (Wright *et al*., 1974).

Lisiak and Vandevelde (1979) reported polio-encephalomalacia in CD infected animals. They observed serious neurological signs in CD affected dogs, such as seizures, chewing fits, depression, myoclonus, head tilting, moving in circular motion, blindness etc.

Vandevelde *et al.* (1980) studied encephalitis symptoms in mature dogs affected with chronic CD. Loss of vision was one of the most important finding associated with the infected dogs. Systemic signs were not observed in old dogs.

Moller *et al.* (1993) categorized CD associated clinical signs into 3 forms, catarrhal form, nervous form and hard pad form. The catarrhal form is associated with inflammation of the conjunctiva, respiratory disorder and gastro-intestinal related disorders. CNS is affected in nervous form showing signs of myoclons, ataxia, and tremor. Increased keratin deposition is noticed in footpads and nose, in hard pad form of the CDV disease.

Maeda *et al.* (1994) noticed skin lesions in CD affected dogs. There was hyperkeratosis and parakeratosis of the nose and foot pad, called as hard pad disease. There was vesicle, pustule and papule formation in and around nose mucous membrane of mouth and eye.

Summer and Appel (1994) noticed signs that were observed in recently affected dogs. There was high rise of temperature, muco-purulent discharges from eye, conjunctivitis, pneumonia and inflammatory nasal mucosa. Gastro-intestinal disorder symptoms were also observed.

Bittegeo *et al*. (1995) observed dental irregularity in young puppies affected with CDV. They observed a wide range of signs related to abnormalities of teeth. Impaction of the teeth, enamel hypoplasia along with hypoplasia of the dentine was reported. Oligodontia was also noted in mixed breed puppies of Tanzania. These pups those were having dental abnormalities didn't had any other systemic disorders.

Vandevelde and Zubriggen (1995) found the neurological signs and studied about the neurological disorder associated with CDV. They stated that CDV attacks the nervous system the virus multiplies in the neurons and glial cells of the brain. Severe immunosuppression is noticed when virus multiplies at the white matter of the brain. The virus remains in the CNS for a long time.

Tiploid *et al.* (1995) studied the diseases related to nervous systems. 220 dog's medical history was noted down showing neuronal disorder signs. 28/38 dogs had non inflammatory CD encephalitis; these dogs manifested extra neuronal signs of increase

in temperature, conjunctivitis, gastroenteritis and respiratory signs. Hyperkeratosis was also noticed in nose and footpad. In case of inflammatory CD encephalitis the symptoms and signs noted were involuntary twitches in a group of muscles, leg muscles, facial muscles etc. Tremor was noticed in 14/84 dogs, myoclonous in 36/84 dogs.

Thirunavukkarasu *et al.* (1996) noted ocular signs in CD affected dogs. Ocular manifestations were noticed in 9 dogs suffering from CD disease. Ophthalmic examinations of these 9 dogs were conducted. Clinical examinations revealed congestion of ocular mucous membrane, mucopurulent discharges from the congested eye. Ophthalmoscopy revealed that the affected dogs were having chorio-retinal lesions with hyper-reflectivity. The study stated that ophthalmoscopy was more routinely used technique for determinations of ocular lesions. The ocular fundus of clinically affected dogs with CD showed vascularity.

Kommonen *et al*. (1997) noted clinical signs related to CD in clinically affected dogs. They observed inappetence, and high rise of temperature between 39.2$^{o}$c to 40$^{o}$c. Respiratory sings observed were rhinitis, tracheitis, bronchitis and pneumonia. Gastroenteritis was noted in dogs recently affected with CD. Early stages showed gastroenteritis in dogs which lasted for 2 days. Animals were depressed and anorectic. Nasal and ocular discharges were noticed. Discharges in chronic form became mucopurulent. Hyperkeratosis of nose and footpad was noticed. CNS signs were observed. Skin form of CD was also observed. Skin was hyperkeratised, vesicles developed on the ear flaps. Vesicular stomatitis was also observed.

McCandlish (1999) stated that it was difficult to say whether the animal suffering from CD will develop neurological signs and symptoms or not but if the nervous form of disease occurs then the affected animal will be more at risk.

Lorenz and Kornegaya (2004) reported that canine distemper virus was most dangerous and common infectious viral disease causing seizures in the affected animals.

Ford (2010) reported clinical signs and symptoms of Rubarthe disease might be limited to the upper respiratory tract. Coughing and nasal discharge are symptoms of this disorder. Puppies having no vaccination record are the most susceptible to this

disorder. It includes signs such as pneumonia, diarrhea, anorexia, dehydration, convulsions. The early sign of this disease is vomition which is the most common finding of this disorder.

Amude *et al.* (2010) described the clinical and neurological signs of Rubarthe disease infection in dogs. Occurrence of neuropathological lesions and nervous infection by CDV may cause various neurological signs.

Machida *et al*. (1992) described clinical signs and symptoms in free ranging veiled palm civet include the dehydration, convulsions, chorea, dyspnea, serous ocular discharge, nasal discharge, diarrhea and local alopecia.

In ursids the clinical signs and symptoms consists of the purulent ocular discharge, nasal discharge, anorexia, vomition, diarrhea, ascending paralysis, profuse salivation and in some cases terminal seizures and coma (Deem *et al*., 2000).

In raccoons clinical signs and symptoms mostly are similar to the domestic dogs but cystitis with pyruia is more frequent and there is jaundice associated CDV is seen in raccoons (Kilham *et al*., 1956).

The dental irregularity may be seen in young puppies affected with CDV (Bittegeo *et al*., 1995).

The young dogs may reveal metaphyseal osteosclerosis of long bones (Baumgartner *et al*., 1995).

Scgliarini *et al*. (2003) studied that dogs with nervous manifestation usually die, but some might recover and may show lifetime residual signs such as a continual myoclonus.

CDV infected monkeys initially showed measles like symptoms including respiratory signs, anorexia, nervous signs, fever and red rashes over the whole body with reddening and inflammation of footpads, conjunctivitis and thick mucoid ocular and nasal discharge (Qiu *et al*., 2011).

In domestic ferrets and black footed ferrets the infection has high fatality rate and clinical signs include the ocular and nasal discharge, diarrhea, anorexia, seizures,

tonoclonus and myoclonus. Black footed ferrets often have stern hyperkeratosis of the foot pads whole body erythematic and rash over chin and groin associated with pruritis (Carpenter *et al*., 1998 and Williams *et al.*, 2003).

**2.12 Histopathological studies**

In experimental studies of CDV in canines, there is severe lymph reduction and necrosis in the cortical zone of the lymph nodes and thymus, white pulp of the spleen and bone marrow (Dungworth, 1993) by 6 to 9 days post-infection.

Koutinas *et al*. (2002) studied the changes include the neuronal deterioration and necrosis, spongiosis due to demyelination, gliosis, mononuclear perivascular cuffing and leptomeningitis. Neuronal necrosis may be present in the cerebral and cerebellar cortex, medullary nuclei and spinal cord.

The selective infection of keratinocytes in the stratum spinosum might be the main incident for the advance of hard pad disease in the canine (Koutinas *et al*., 2004).

Pardo *et al*. (2005) described the post mortem lesions in dogs affected with CDV and found demyelination of the white matter of the cerebellum and necrosis, and lymph depletion. Within the areas of necrosis in neurological tissues, astrocytes and glial cells enclosed droplets like 2-5 micrometers eosinophillic intranuclear or intracytoplasmic viral inclusions. Lymphoid depletion and necrosis in the white pulp of the spleen were seen.

Noleto *et al.* (2011) found lentz bodies in the white blood cells of 10 day old puppies having signs confirmatory to CDV. The pup had history of seizures of a time lapse of 10-20 minutes each. They stated that lentz bodies can be considered as a tool for early diagnosis of CD. Increase of lentz bodies were seen in viremic stage. Intracytoplasmic corpuscles were observed in histopathology.

Daga *et al.* (2012) reported that there is decrease in the lymphocyte number at initial stages of CD followed by decreased in number of white blood cells and the reason behind this phenomenon can be related with multiplication of the CDV in the lymph node.

Chvala *et al.* (2007) reported histo-pathological findings in CD affected animals. They found out fibrinous pneumonia with necrosis of the lungs. They found

intracytoplasmic inclusions bodies and acidophilic intranuclear inclusion bodies in the lung tissues.

Cho *et al.* (2015) reported CDV illness in a female wild racoon dog, Korea. The animal had a history of generalized seizures and mild decrease in the concentration of white blood cells. Post mortem revealed hemorrhages in the nasal cavity and nasal mucous membrane, hemorrhages in the urinary tract, urinary bladder, hematuria, blood was found in the thoracic region Pneumonia was also observed. Histopathology revealed presence of intranuclear and intracytoplasmic inclusion bodies in the cerebellum and epidermal appendages section.

Sousa *et al.* (2015) reported inclusion bodies in the white blood cells and red blood cells in an animal died of CD.

Gebara *et al.* (2004) reported the CNS infiltration of perivascular mononuclear cells in tissue section of brain. Demyelination was a consistent finding.

Amude *et al.* (2006) reported multifocal lesions in the CNS in a 7 months old mixed breed male presented with history of episodic seizures within 1 week and compulsive walking.

Lan *et al.* (2006) reported inclusion bodies in stomach of a CD suspected dogs. The tissue section of the stomach revealed absence of inflammation and encephalitis is seen in 3 dogs that died without showing any clinical signs and symptoms.

Headley and Graca (2000) reported the presence of CDV inclusion bodies in the astrocytes in 82% of affected dogs. Eosinophilic inclusion bodies were observed in the epithelial cells of the urinary bladder, lungs, stomach, kidney and tonsil in a decreasing percentage.

### 2.13 Diagnosis

#### 2.13.1 Samples for collection

Elia *et al.* (2006) studied that lymph tissues, conjunctiva swab, saliva and urine were best samples for detection of CDV by quantitative real time PCR (qRT-PCR). The frontal lobe of the brain was also rich in virus.

Latha *et al*. (2007a) collected 167 conjunctiva swabs and processed for detection of CDV by Dot- ELISA.

Pawar *et al*. (2011) detected CDV from brain tissue by targeting CDV N gene and concluded that saliva swabs would be model samples for RT-PCR based confirmation of CDV genome as it was found that they have similar amount of CDV mRNA as that is revealed in brain of dead canine.

Elia *et al*. (2015) collected blood, conjunctiva and urine samples regardless of clinical stage for CDV detection and isolation and results revealed that urine and rectal swabs would be more beneficial for ante-mortem diagnosis of CDV.

Swati *et al*. (2016), Seshadri *et al*. (2015) and Ashmi *et al*. (2017) detected CDV from ocular, nasal secretions, and from blood.

Fischer *et al*. (2013) detected CDV virus from blood, urine, rectal and conjunctiva swabs. Blood resulted in more positive followed by urine, rectal and conjunctiva swabs.

### 2.13.2 Propagation of virus

Initially, CDV detection and isolation has been conducted by different techniques and in different cells. Chorion-allontoic membrane of embryonated chicken eggs could be used for cultivation of CDV (Ezeibe *et al*., 2005 and Haig *et al*., 1956).

Appel and Jones (1967) described that CDV has been cultivated in canine alveolar macrophages. The attenuated form of CDV could enthusiastically be grown in epithelial and fibroblast cell lines but isolation of virulent CDV in these cells is difficult (Appel, 1978). But all these methods are prolonged and multiple blind passages are required before any proper cytopathic effects of the virus been visible (Swati *et al*., 2016).

### 2.13.3 Serological diagnosis

Moller *et al.* (1993) stated that recently infected animals with CDV develops IgM antibody. ELISA test was used for detection of IgM Ab against CDV.

Koutinas *et al.* (2002) diagnosed Ab against CDV serologically by use of neutralizing serum antibody detection technique.

Loots *et al.* (2018) detected CDV specific Ab titre by using serological tests such as indirect FAT, ELISA and SNT. Indirect FAT and ELISA tests detected IgM and IgG Abs against CDV in domestic and non- domestic canids.

Martinez and Ruiz (2002) used ELISA and indirect FAT diagnostic tools for detection of Abs against CDV in wild canids. The study concluded that indirect FAT can be used as a golden standard test against CDV Ab detection.

Elia *et al.* (2015) reported that N protein-based detection ELISA tests or whole virion-based detection assay should be used for serological diagnosis of CDV.

Vanak *et al.* (2007) used dot ELISA diagnostic kit for detection of IgG and IgM antibody against CDV. Serum samples from 75 dogs were collected and Ab against CDV was detected by using Biogal Immunocomb ELISA kit.

Cha *et al.* (2012) detected CDV Ag by using CDV antigen detection kits. CDV was detected in 14 collected samples. Survey found CDV in 44.1% of the collected samples.

Moller *et al.* (1993) reported for detection of CDV IgM antibodies ELISA technique is used. It adds new outlooks to serological diagnosis of current or recent CDV affections based on investigation of one serum samples where vaccination is regularly done.

Tiploid (1995) reported a study to determine whether clinical data and clinico-pathological data could distinguish inflammatory CNS diseases from diseases of other types. This is used to search for criteria allowing and distinguishing different specific inflammatory diseases. The signalmen, historical findings, extra-neural and neurological signs and neurological lesions contributed not significantly to a specific diagnosis. A countable proportion of canines with canine distemper can be diagnosed based on congregation of their growing ages, lymphopaenia, hyperkeratosis, myclonus, abnormal cerebrospinal fluid (CSF).

Frisk *et al*. (1999) studied that by using real-time PCR. CDV ribonucleic acid can be detected from clinical bio samples like sera, blood components and cerebrospinal fluids from 40 canines suspected for distemper. Analysis was related to clinical results. It is also related to neutralizing anti-CDV antibody titers, PM findings. The specificity of real-time-PCR for finding of CDV is influenced by region specific primers. PCR assay is extremely specific and sensitive approach for the ante mortem diagnosis for canine distemper.

Sen *et al. (*2002) described a study canine distemper can be diagnosed by inclusion bodies in the peripheral blood cells, serum CDV specific antibody titers, CDV antigens in the ocular swabs and enzymatic positivity of blood lymphocytes in the dogs.

Kommonen *et al*. (1997) described a study on occurrence of canine distemper in dogs in Greenland in 1990. An outbreak found in a region with high density of canine population. The dogs were having a history of complete vaccination record. The total numbers of cases were found to be at least 6000. 860 cases were confirmed by diagnostic techniques like indirect fluorescent antibody technique. The samples used for diagnosis of canine distemper were epithelial cells.

Koutinas *et al*. (2002) described a study on 19 random cases. These were having signs of neurological disorder. These were examined before and after euthanasia. The detection of distemper was confirmed by Immunohistochemistry by detecting viral antigens, serologically by neutralizing serum antibodies, histopathologically by detecting distemper inclusion bodies.

Ramadass and Latha (2001) conducted a study on detection CDV. They detected CDV by Dot Enzyme Immunoassay by using conjunctiva swabs of canines suspected for CD infection. The samples were processed for immuno fluorescence. This technique detected 30% of the suspected samples. DIA is an easy, rapid and confirmatory approach for regular diagnosis of CD infection in canine population. Large number of samples can be processed in this diagnostic technique within a short span of time with minimum facilities inside laboratory.

Saito *et al*. (2006) described that canine distemper can be detected by reverse transcriptase Polymerase chain reaction (RT-PCR) from urine sample of suspected

canines having clinical signs of encephalitis. A part of gene of CDV was magnified from urine bio-samples 20 affected dogs. Among them 10 asymptomatic dogs bio-samples were found to be negative by RT-PCR. In RT-PCR urine samples was found to be more specific and sensitive than serum, CSF. CSF was found to be least sensitive. CSF was used to screen the suspected dogs having severe clinical signs like neurological signs and extra- neural systemic signs. They suggested that RT-PCR may be conducted for rapid and confirmatory diagnosis of CD suspected dogs. PCR assay is the gold standard diagnostic tools for canine distemper screening.

Amude *et al*. (2008) described that PCR assay is the best for diagnosis of canine distemper suspected dogs. They conducted study on CD suspected dogs using saliva swabs. 67 % of suspected dogs were found positive by conventional PCR assay. Abnormalities in hematological findings may be suggestive for canine distemper diagnosis. But it may not be specific and confirmatory. Abnormalities in CSF might be suggestive for canine distemper virus (CDV) infection by lymphocytic pleocytosis.

Galan *et al.* (2014) used indirect ELISA as a diagnostic tool for detecting antibody against CDV. The study reported the presence of high level of serum Ab against CDV in unvaccinated dogs.

Temilade *et al.* (2015) used rapid Ab detection kit to detect CDV antigen in Nigeria. Blood samples were collected from 40 dogs out of these some dogs were vaccinated and some dogs were not vaccinated. 3/40 dogs were having CDV antibody.

Latha *et al.* (2007) used Dot ELISA technique along with indirect immunofluoresence technique to investigate the CDV disease in vaccinated and unvaccinated dogs. They also stated that IFA as a universal accepted diagnostic test for CDV.

Li *et al.* (2013) stated that Dot ELISA is a quick and very trusted test to detect CDV Ag as compared with simple ELISA.

### 2.13.4 Immunohistochemistry

Gathumbi *et al.* (1993) used immune-histo-chemical for diagnosis for CD antigen in tissue fixed in formalin and tissue embedded in paraffin. This study stated that IF technique can be used for CD by making smears of conjunctiva content.

Haines *et al.* (1999) and Van Moll *et al.* (1995) used immune-histo-chemical for diagnosis for CD antigen in wild carnivores, Germany.

Macs *et al.* (2003) stated CDV can be detected immune-histo-chemistry. Monoclonal Ab was commercially available and this Ab was used for immune-histo-chemistry.

Fairley *et al*. (2015) reported the outbreak of CD after vaccination. Encephalitis was detected at post mortem in 2 Border coolie breeds. Diagnosis was carried out of immune-histochemistry.

Koutinas *et al.* (2002) diagnosed Ab against CDV serologically by use of neutralizing serum antibody detection technique. Immuno-histochemistry was also employed for diagnosis of CDV.

### 2.13.5 Molecular assays

#### 2.13.5.1 Nucleic acid detection by PCR

The dawn of molecular methods brings diagnostic techniques that are outstanding with regard to sensitivity and specificity (Martella *et al*., 2008 and Soma *et al*., 2013).

Polymerase chain reaction (PCR) and nucleic acid hybridization research work using single stranded RNA probes have been achieved to identify virulent virus in tissue culture and histology section (Zurbriggen *et al*., 1993).

Reverse transcription has been used to find out CDV RNA in buffy coat cells from dogs with chronic CDV infection. Nucleoprotein and RNA of CDV has been found by reverse transcription- polymerase chain reaction (RT-PCR) using ocular swab, serum, whole blood, saliva swab and CSF from dogs with distemper (Frisk *et al*., 1999).

To increase the efficiency of detection and isolation of CDV in samples with reduced viral load, the RT-PCR has been revealed to be the most effective method of CDV diagnosis (Kim *et al*., 2001).

Reverse-transcription PCR, nested-PCR, and southern blot hybridization has been made for use for the revealing of the phosphoprotein gene of CDV in peripheral

blood mononuclear cells and various internal organs of canines and fur animals in Poland (Rzezutka and Mizak, 2003).

Sequence determination using this approach may yield molecular epidemiologic information concerning vaccine efficacy (Stanton *et al*., 2002).

During outbreak of CDV in Alaska, PCR was used to detect the infectivity and detect the origin of the responsible strain to Siberia (Maes *et al*., 2003).

Viral mRNA has been found out in footpad specimens from affected dogs (Grone *et al*., 2003).

Shin *et al*. (2004) studied that there was a 15 % rise in detection CDV in urine sample when RT-PCR combined with Nested- PCR. Regardless of the period and form of distemper, a positive result was highly unambiguous for diagnosis.

The assay displayed high specificity and a quantitative TaqMan was authenticated on clinical samples, including many tissues and organs collected from dogs naturally infected by CDV (Elia *et al*., 2006).

Semi-nested PCR has also been used to competently detect CDV in paraffin embedded nervous tissue. One of numerous techniques that have been used for the detection of CDV is the RT-PCR. Nucleocapsid protein of CDV was amplified from the conjunctiva samples of distemper suspected dogs and cloned into PRSET B vecto (Latha *et al.*, 2007a).

Recently, a hemi- nested multiplex PCR which offers a speedy loom for the study of CDV outbreaks has been described. The system was used to genotype most important CDV lineages and is supported to be helpful for large scale molecular epidemiological studies of CDV and for the screening of vaccine related disease (Martella *et al*., 2007).

Pawar *et al*. (2011) tested the presence of CDV in ocular and saliva swabs and brain tissues by RT-PCR. Quantitative real time PCR was used to enumerate the H gene expression in ocular swabs and brain tissues sample using SYBR green chemistry and described that QRT-PCR could reinstate the conventional RT-PCR for clinical diagnosis of CDV in due course.

Fischer *et al*. (2013) found and differentiated the field and vaccine strains of CDV using reverse transcription followed by nested real time PCR (NRT-PCR) in Brazil. They used the Msp-I restriction enzyme to discriminate the field and vaccine strains by restriction fragment length polymorphism (RFLP) study.

Romanutti *et al*. (2016) found the full length F protein of CDV from domestic dogs in Argentina by using RT-PCR.

Tozato *et al*. (2016) engaged the real time RT-PCR for CDV detection. CDV was established by RT-PCR and further amplified H and L genes cloned, sequenced and phylogenetically analyzed (Swati *et al.*, 2016).

Nobleza *et al*. (2017) isolated and characterized the CDV H gene from domestic dogs by using nested NRT-PCR.

Ashmi *et al*. (2017) detected CDV N gene by using RT-PCR and Nested PCR N gene positive samples were put forward for amplification of H and F genes.

**2.13.6 Gene sequencing**

Lan *et al*. (2006) compared the sequences and phylogenetic tree was constructed with the three CDV isolates if vaccinated dogs with sequences of commercially available vaccine strains. The new isolates of CDV joined to the clades of the Asia-1 group that was far from the vaccine group.

Simon- Martinez *et al*. (2008) detected the genetic variant of CDV from clinical cases in two vaccinated dogs in Mexico by sequence analysis of field strains. The sequence obtained was dissimilar to that of vaccine sequences. They were closely related to USA and Germany. The results revealed that novel CDV may be present in Mexico.

Woma *et al.* (2010) phylogenetically evaluated the wild type CDV strains of South Africa with the other strains from gene bank. The results revealed that all South African isolates showed a separate African Clade of their own.

Pawar *et al*. (2011) sequenced the N gene and compared with CDV isolated from other countries and found 89-98 % identity. A phylogenetic tree was drawn with

the aligned sequences revealed a distinct Clade for the two Indian viruses while the other isolate was found to be more deviating from other CDV. The commercial vaccine strain grouped with CDV from USA, Taiwan, Switzerland and China and was divergent from other Onderstepoort vaccine strain.

Seshadri *et al*. (2015) established that phylogenetic tree by comparing gene sequence of the field isolates in Tamil Nadu with that of reference sequences available in Gene bank. The results revealed that the Onderstepoort vaccine strain showed 91 % and 89 % homology with H gene and F gene of the field isolates respectively. There was only a homology of 95 % between the field isolates and the R252, USA strain. All the strains from Japan showed similar homology with the Tamil Nadu field isolates. The H gene of local strain shows more homology with the reference strains than the F gene.

Swati *et al.* (2015) sequenced the field sequenced the field isolates of CDV specific to F, M and P genes and constructed the phylogenetic tree by subjecting sequences to multiple sequence alignment using Clustal-W method. The results revealed that the F gene of Indian isolates showed 91.4 – 96.1 % nucleotide homology with other 32 known CDV strains.

Swati *et al*. (2016) compared the nucleotide sequences of H and L genes of CDV from Ludhiana with that of other 22 CDV strains available in NCBI database along with vaccine strains. The CDV strains from different geographical locations were used to construct the phylogenetic tree using maximum likelihood method in MEGA 6.06. The results revealed that the local isolates of H gene CDV revealed 91.9 – 95.9 % sequence homology with that of other 21 CDV strains, where as L gene of CDV revealed 94.8- 98.1 % nucleotide sequence homology.

Romanutti *et al*. (2016) studied the full length F protein sequences of CDV strains circulating in Argentina. A neighbor joining phylogeny tree was built to detect the evolutionary relationship between the 15 CDV Argentina strains were closely related to each other sharing the SA2 Clade (divergence of 1.1 % among them) which had an evolutionary divergence of 8.8 % with respect to the vaccine Clade.

Ashmi *et al*. (2017) analyzed the nucleotide sequences of field strains in Tamilnadu with the other CDV nucleotide sequences available in Gene bank.

Phylogenetic tree of the partial H and F gene nucleotide sequences were built using Maximum likelihood method. They concluded that field CDV viruses were divergent and varied from the vaccine strains. The field strains were revealed as a separate clade in the phylogenetic tree and fall under European lineage.

Nobleza *et al*. ( 2017 ) reported the sequence analysis of field CDV strains and concluded that field strains revealed 96 % maximum homology to the Rockborn strain, Brazil strain and lesser with the China. The phillipine strains were more similar to the Thailand strains.

Espinal *et al*. (2018) showed that there were three divergent CDV lineages circulating in Columbia South America by Phylogenetic analysis.

Loots *et al*. (2018) examined the genetic diversity of South African wildlife CDV isolates using the hemagglutinin gene and it revealed elevated mark of homology to CDV in dogs of SA. Phylogenetic analysis results established the existence of 12 geographical lineages with CDV strains from SA wildlife falling within the SA lineage.

### 2.13.7 Hematobiochemical changes

Tiploid (1995) reported that lymphopaenia (100-800 lymphocytes/µl) was most frequently found in dogs with distemper encephalitis. Most of the serum biochemical abnormalities were non- specific.

Greene and Appel (1998) studied that in experimentally infected animals, frequently hematologic findings were lymphopaenia, sometimes associated with increased or decreased leucocytes with increased number of immature neutrophils, anemia and decrease in platelet count.

Greene and Appel (2006) described that abnormal hematological findings in canine distemper infection included a complete decrease in lymphocytes accompanied by destruction of lymphoid tissues. This finding continues to be seen in juvenile dogs. These alterations are followed by systemic and neurologic alterations. Total platelet count <30,000 cells/µl. Decrease in Hb concentration is noted only in experimentally inoculated CDV but not a very notable finding in older or naturally acquired Total protein analysis in serum of CDV infected neonates included decreased albumin and

increased α and γ globulin concentrations. Some puppies infected prenatally or neonatal with CDV have marked hypoglobulinemia from persistent immunosuppression caused by the virus.

Kumar (2011) carried out hematological and biochemical studies on 18 cases of canine distemper. Decreased in lymphocyte count was observed in 10 dogs but the TLC was found to be normal. 4 dogs showed decrease in lymphocyte no. Eosinopenia, monocytopenia were observed along with decreased RBC count and anemia. There was decrease in PCV. Among serum enzymes, there was a marked rise in AST values in old dog encephalitis dogs. There was marked rise in serum aspartate amino transferase concentration.

Lal *et al.* (2011) studied hematological studies on 10 canine distemper affected dogs. Hematological parameters viz Hb, PCV, TEC, TLC revealed marked rise before treatment with decrease up to control values had been seen after treatment. A marked decrease in total platelet count was seen in 1$^{st}$ day of treatment, and then noteworthy increase was seen on 12$^{th}$ day of treatment but lower than healthy control group. Differential leucocytic count showed significant increase in neutrophils and decrease in lymphocytes and monocytes were observed on 1$^{st}$ day of treatment which decreased and increased, respectively on 5$^{th}$day of treatment.

Saini *et al.* (2011) carried out hematological studies on five dogs of age between 3 to 4 months suffering from canine distemper. Canine distemper infected dogs on first day had considerably higher values of hemoglobin, total erythrocyte count, packed cell volume and total leucocyte count as compared to healthy control. These values reduced drastically on fifth day of treatment in these cases and appeared near to healthy control.

Daga *et al.* (2013) conducted a study showing hematological alterations in canine distemper affected dogs. Hb concentration was 10.44 g% with alteration of 2 g%, PCV of 30.46±4.25. There was decrease in RBCs concentration.

Behera *et al*. (2014) conducted a study to evaluate hematobiochemical parameters in 10 dogs with canine distemper virus (CDV) infection. Hematology showed significant (<0.05) decrease in the level of hemoglobin, RBC, hematocrit and thrombocytic count in 70 per cent of cases indicative of normocytic hypochromic

anaemia as well as thrombocytopenia and neutrophilic leucocytosis with lymphopaenia in 80 per cent of cases. There was also occasional presence of intracytoplasmic inclusion bodies both in RBC and WBC in 20 per cent cases. Plasma biochemistry showed mild hypoproteinemia and hyperglobulinemia with significant hypoalbuminemia.

Willi *et al*. (2015) studied hemato- biochemical profile in 15 dogs suffering from canine distemper. The hematological profile revealed decreased in Hb concentration in nine out of thirteen dogs. Decrease in TLC in 8 out of 62 dog increases in monocytes in 5 out of 11 cats. Blood biochemistry results showed only non-specific changes in the dogs.

# MATERIALS AND METHODS

Present study was carried out in the Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary Science and Animal Husbandry, Odisha University of Agriculture and Technology (OUAT), Bhubaneswar, Odisha during the period from June 2019 to July 2020.

### 3.1 Source and criteria of animals

Pups presented in the Teaching veterinary clinical complex and peripheral veterinary dispensary of Bhubaneswar city with the signs of fever, posterior weakness, convulsion, profuse salivation and history of no vaccination were clinically examined**.**

### 3.2 Collection of blood samples

After properly restraining the animals, blood samples were collected taking all the aseptic precautions. About 1ml of blood was taken in sterile vials containing disodium salt of ethylenediaminetetraacetic acid (EDTA, 1mg/ml) from recurrent tarsal vein. Samples with EDTA were used for hematological studies.

### 3.2.1 Hematological Examination:

Hematological studies were done on vet auto hematology analyzer.

**Table 1. Hematological parameters estimated and their units**

| Sl. No. | Parameters | Units |
|---|---|---|
| 1 | Hemoglobin (Hb) | Gram per deciliter (g/dl) |
| 2 | Packed cell volume (PCV) | Per cent (%) |
| 3 | Total Leukocyte count (TLC) | Thousands per microlitre ($\times 10^3$/µl) of blood |
| 4 | Total Erythrocyte count (TEC) | ($\times 10^6$ / µl) of blood |
| 5 | Differential Leukocyte count (DLC) | Per cent (%) |

### 3.3 General laboratory reagents and materials

Analytical and molecular biology grade chemical reagents used for the preparation of all solutions and buffers were obtained from a number of suppliers including Qiagen (Germany), Sigma Aldrich (USA), Himedia Laboratories Pvt. Ltd. (India), Invitrogen (USA), Geni (India), Biocon (India) and Promega (USA) . All aqueous solutions were made using double glass distilled water. These were disinfected by autoclaving at $121^0$ C for 15 minutes.

#### 3.3.1 Glassware

Glassware such as conical flasks, pipettes, test tubes etc made up by pyrex and Kontes brand were used during the research work. Glassware was bathed in basic detergent (Persil) whole night. Then these instruments were scrubbed and individual items were washed properly under flowing tap water. Individual item was rinsed with non-ionized water followed by single distilled water. Finally all the materials were rinsed with double distilled water. These were air dried, packed. These were sterilized in hot air oven at $160^0$ C for 2 hours.

#### 3.3.2 Plastic ware

Micro centrifuge tubes (0.2ml, 0.6ml, 1.5ml and 2ml), centrifuge tubes (15ml and 50ml), micropipette (10 µl, 100 µl, 200 µl and 1000 µl), storage vials (5ml and 10ml) from Tarsons were used throughout the work. Disposable sterile 2ml, 5ml and 10ml syringes, EDTA vials, clot activators vials were used in the study.

### 3.4 Samples

In the present study saliva and ocular swabs were collected from a total of 10 CD suspected dogs from Teaching Veterinary Clinical Complex, College of Veterinary Science and Animal Husbandry, Odisha University of Agriculture and Technology (OUAT), Bhubaneswar, Odisha, Peripheral Veterinary Dispensary of Bhubaneswar City.

Saliva and ocular samples were collected in a 2ml ependroff tubes and were kept in ice- pack container. Blood samples from Cephalic vein were collected in EDTA vials and also kept in ice-pack container. All collected samples were brought to laboratory for further processing.

### 3.4.1 Sample processing

Saliva and ocular swabs collected from suspected dogs in sterile Phosphate Buffer Saline (1X PBS) in eppendorf tube were squeezed properly and centrifuged at 5000 rpm for 5 minutes in a centrifuge at 4 $^{0}$C. Then samples were stored at -80$^{0}$ C.

## 3.5 Molecular diagnosis of canine distemper virus by cDNA synthesis and polymerase chain reaction (PCR)

### 3.5.1 PCR kit

Thermo scientific Verso cDNA synthesis kit (RT-PCR kit) was used, which contained the following reagents cDNA synthesis buffer (4 µl), dNTP mix (2 µl), reverse transcriptase enzyme /verso enzyme (1 µl), RT enhancer (1 µl), Oligo (dT) primer /random hexamer (1 µl), template (5 µl), nuclease free water.

### 3.5.2 Primers

The primers used for the amplification of N gene (243 bp) of CDV were given below

1622 (F) - (ACCTGGGACCAGTGAAGAGA) HPSF 0.01 scale

1864 (R) - (TCTCTCTGAGGGCTTTGAGG) HPSF 0.01 scale

HF- (AACTTAGGGCTCAGGTAGTC) 0.05 scale PAGE

HR-(AGATGGACCTCAGGGTATAG) 0.05 scale PAGE

### 3.5.3 Materials for RNA extraction

The following reagents were used in the extraction of viral RNA from samples

1. Buffer AVL containing carrier RNA
2. Ethanol ( 96-100% )
3. Buffer AW1
4. Buffer AW2
5. Buffer AVE

### 3.5.4 Other materials/ reagents for PCR

**Table 2. Reagents/materials used for PCR**

| Sl. No. | Material | Purpose |
|---|---|---|
| 1 | Micro centrifuge tube | For RNA extraction |
| 2 | PCR tubes | For cDNA synthesis and PCR |
| 3 | Agarose | For preparation of 1.5 % gel |
| 4 | Ethidium Bromide | For staining gels at a final concentration of 10µg/ml |
| 5 | DNA Marker | For studying the migration pattern of DNA specific to targeted N gene |

### 3.5.5 Procedure

#### 3.5.5.1 Extraction of viral RNA

The RNA was extracted from sample using Viral RNA extraction minikit as per manufactures instructions

1. The amount of buffer AVL containing carrier RNA was 560 µl. It was pippetted out into a 1.5 ml micro centrifuge tube.
2. The amount of sample was 140 µl. Addition of sample to the buffer AVL carrier RNA was done in the micro centrifuge tube. Mixing of mixture solution was done by pulse-vortexing for 15 seconds.
3. Incubation of the mixture was done at room temperature (20 $^{0}$ C) for 10 minutes.
4. Centrifugation of the mixture tube was done to remove drops from the inside of the lid.
5. The amount of ethanol (100% molecular grade) was 560 µl. It was put into the sample. Mixing of mixture solution by pulse-vortexing was done for 15 seconds. Centrifugation of mixture tube was done to expel out drops from the inside the lid after proper mixing.
6. The amount solution was 630 µl. It was applied to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Centrifugation of the tube was done at 8000 rpm for 1 minute after properly closing the cap. QIAamp Mini column was put into a clean 2ml collection tube and the tube containing the filtrate was discarded.

7. QIAamp Mini column was opened and step 6 was repeated until all of the lysate had been loaded onto the spin column.
8. Addition of 500 µl of buffer AW1 was done after opening mini column. Centrifugation of the mixture column was done at 8000 rpm for 1 minute after properly closing the cap. Mini column was placed in a clean 2 ml collection tube and the tube containing the filtrate was discarded.
9. 500 µl of buffer AW2 was added after opening mini column. Centrifugation was done at full speed 14000 rpm for 3 minutes after closing the cap.
10. QIAamp Mini column was placed in a clean 1.5 ml micro centrifuge tube. The old collection tube containing the filtrate was discarded. Addition and equilibration to room temperature of 60 µl of buffer AVE was done after opening mini column. Incubation at room temperature for 1 minute was done after closing the cap.
11. Centrifugation of mixture was done at 8000 rpm for 1 minute. Elution yielded 100% viral RNA.
12. Viral RNA was stored at $-80^0$ C.

**3.5.5.2 Synthesis of cDNA**

The synthesis of cDNA was carried out using thermo scientific verso enzyme cDNA synthesis kit following manufactures instructions.

1. Mastermix was prepared adding 4 µl cDNA synthesis buffer, 2 µl dNTP, 1 µl RT enhancer, 1 µl OligoDT /Random hexamer, 1 µl Verso enzyme, 6 µl nuclease free water.
2. 15 µl of master mix was added to 5 µl of viral RNA making a total volume of 20 µl.
3. The sample was transferred to a thermo cycler incubator and reverse transcription was carried out at $42^0$ C for 30 minutes for activation and cDNA synthesis and $95^0$C for 2 minutes for inactivation of reverse transcription enzyme .
4. The synthesized cDNA was stored at $-80^0$ C for further use as template for PCR.
5. The newly designed primer and referred primer used for the amplification of N gene (243 bp) of CDV was given below

1622 (F) - (ACCTGGGACCAGTGAAGAGA) HPSF 0.01 scale

1864 (R) - (TCTCTCTGAGGGCTTTGAGG) HPSF 0.01 scale

HF- (AACTTAGGGCTCAGGTAGTC) 0.05 scale PAGE

HR-(AGATGGACCTCAGGGTATAG) 0.05 scale PAGE

### 3.5.5.3 Polymerase chain reaction

PCR amplification was optimized in 25 µl PCR reaction mixture. The composition of mixture prepared is provided below;

**Table 3. Composition of PCR reaction mixture**

| Sl. No. | Reagents | Quantity (µl) |
|---|---|---|
| 1 | Master mix | 12.5 |
| 2 | Forward Primer | 1 |
| 3 | Reverse Primer | 1 |
| 4 | cDNA | 5.5 |
| 5 | Nuclease free water | 5 |
| | Total | 25 |

**Table 4. Temperature, time and cycles of events in PCR**

| Sl. No. | Events | Temperature | Time | |
|---|---|---|---|---|
| 1 | Denaturation | $95^0$C | 5 minutes | 1 cycle |
| 2 | Denaturation | $95^0$C | 30 seconds | 35 cycles |
| | Annealing | 55-$60^0$C | 1 minute | |
| | Extension | $72^0$C | 1 minute | |
| 3 | Extension | $72^0$C | 10 minutes | 1 cycle |
| | | $4^0$C | ∞ | - |

### 3.5.5.4 Analytical agarose gel electrophoresis

Gel electrophoresis was carried out to analyze the PCR amplicon. Agarose 1.5 % was prepared using 1× TAE buffer (pH 8.6 by melting and allowed to cool to $50^0$C. A final concentration of 0.5 µg/ ml of Ethidium bromide was added to the molten agarose and mixed thoroughly. The molten agarose was casted in a tray fitted with comb. After solidification, the comb was taken out and the gel is placed into the

submarine horizontal electrophoresis chamber filled with 1× TAE buffer sufficient to immerse the agarose gel.

About 5 µl of PCR product and 4 µl of 100 bp DNA ladder was loaded as molecular marker in all lanes. Electrophoresis was carried out at the rate of 5V/cm until the tracking dye (Bromophenol blue) passed out of the gel.

### 3.6 Sequence analysis

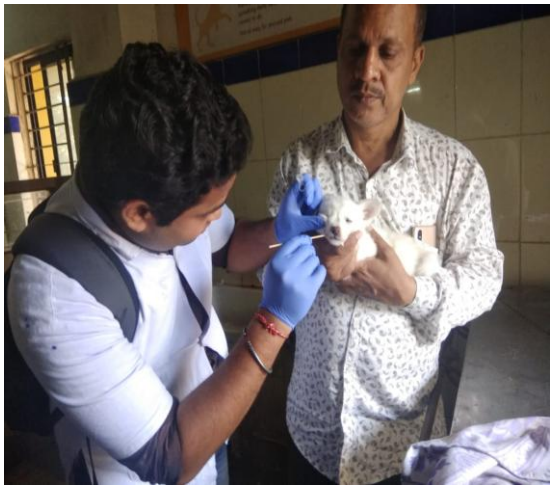
#### 3.6.1 Sequencing

The sequencing of RT-PCR products (20 µl) specific to N gene of field samples was carried out in ABI 3730 DNA Analyzer and with chromas lite software.

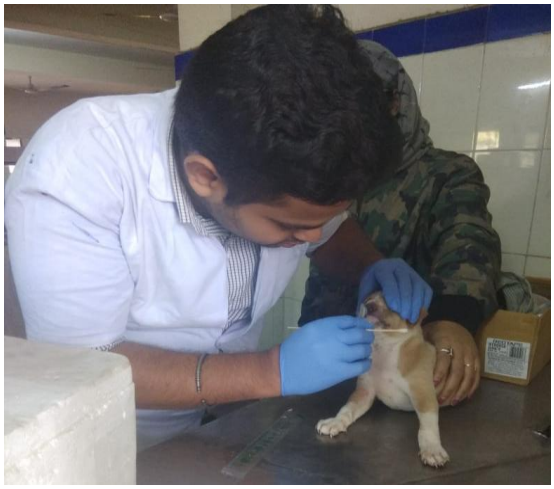
#### 3.6.2 Analysis of nucleotide sequencing

By the help of BioEdit Sequence Alignment Editor (version 7.2.5) the sequences were screened to produce sequence analysis data. Multiple sequence alignment, sequence homology matrix for the nucleotide was the components used for sequence screening.

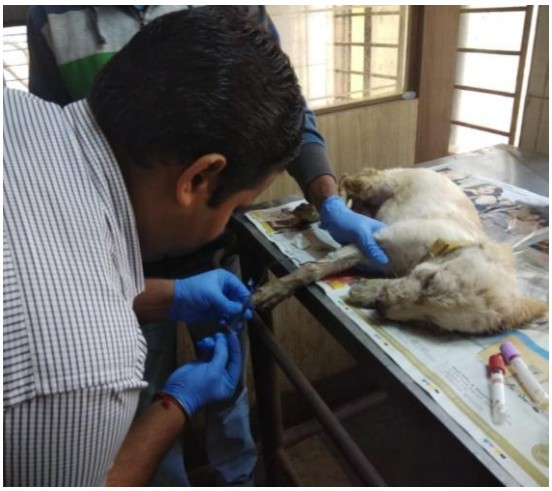
Consent sequence for N gene was produced using the forward and reverse sequence data. Sequence identity search against NCBI-gene-Bank database (http: www.ncbi.nlm.nih.gov/genbank) was done using the Basic Local BLAST algorithm. Many sequences of N gene were retrieved for comparison with the local sequences from the sequences thrown up during sequence identity search using BLAST in gene-Bank.



**(a)**

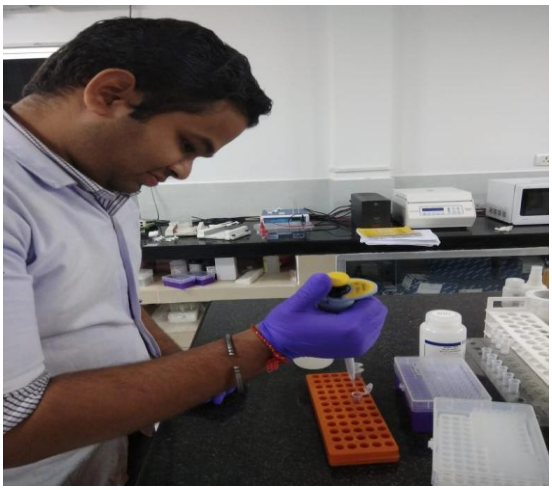


**(b)**



**(c)**

**Fig. 1(a-c): Collection of saliva swab, ocular swab and blood bio-samples in canine distemper suspected cases**



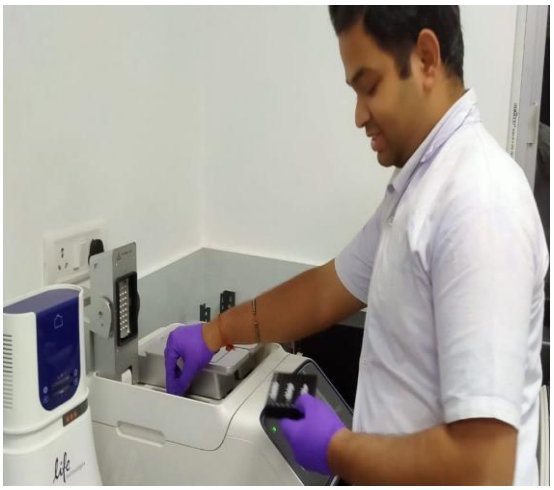


**Fig. 2: Processing of saliva and ocular swabs samples in the laboratory**

# RESULTS

## 4.1 Clinical examination

Ten pups clinically suspected for canine distemper (CD) were included in the present study during the period that stretched from June 2019 to July 2020.Of the 10 pups, 04 pups were presented in the teaching veterinary clinical complex, OUAT and rest 06 were from peripheral veterinary dispensary of Bhubaneswar city. The information gathered during clinical examination of such pets is depicted in the table 5 and 6. Description and history of the pet dog i.e., breed, sex, age, primary vaccination status, clinical signs, type of samples collected were recorded for the study. All the pups were below 71 days old, 05 pups were below 45 days. There were 02 local non-descriptive breeds and rests 08 were recognized breeds. Numbers of male pups were 07 and female pups were 03. Except one pup, none of the pups were vaccinated against CD. One pup was vaccinated at the age of 45 days with a combined vaccine of canine distemper and canine parvo and presented 3 days post-vaccination.

## 4.2 Detection of canine distemper virus (CDV)

Viral RNA was extracted from the 20 bio-samples saliva (n=10) and ocular (n=10). Discharges of 10 pups were included in the study. The designed primers (1622 (F) - (ACCTGGGACCAGTGAAGAGA) HPSF 0.01 scale, 1864 (R) - (TCTCTCTGAGGGCTTTGAGG) HPSF 0.01 scale)targeting N gene could detect CDV through cPCR in 8 samples of 4 pups (4 saliva and 4 ocular).Of the 04 dogs, 01 was from the peripheral veterinary dispensary of Bhubaneswar city and rest 3 were from TVCC, OUAT. The referred primer (HF- (AACTTAGGGCTCAGGTAGTC) 0.05 scale PAGE, HR-(AGATGGACCTCAGGGTATAG) 0.05 scale PAGE) failed to detect CDV. The size of PCR products of N gene by 1.5% electrophoresis was 243 bp (Fig.3).

## 4.3 Sequence analysis of N gene

The sequences generated for N gene were compared with other CDV sequences available in NCBI- GenBank. The obtained nucleotide sequences of CDV

N gene were subjected to BLAST analysis for comparing sequences homology with other CDV reference strains from different geographical areas present in NCBI data base. The obtained nucleotide sequence of CDV N gene was found 97.13% homology with other CDV reference strains in NCBI data-base.

### 4.4 Hematological evaluation

The hematological values of the CDV infected pups are presented in the table 7. The mean ± SE values of hemoglobin (Hb), packed cell volume (PCV), total erythrocytic count (TEC) and total leucocytic count (TLC) were recorded as 8.87 ± 0.52 g/dl, 26.72±1.41 %, 4.57±0.23×$10^6$/µl and 16.51±1.34×$10^3$/µl, respectively. Differential leucocyte count revealed 66.80±0.92% neutrophils, 26.80 ± 1.08 % lymphocytes, 3.70 ± 0.30 % monocytes and 2.70 ± 0.26% eosinophils.

The total erythrocyte count (TEC), hemoglobin (Hb), packed cell volume (PCV) and platelet count (THR) were found lower than the standard range. Earlier workers opined that, persistence of the virus in the bone marrow causes erythriod hypoplasia, results hematological alterations. The consequence of viral persistence in bone marrow has been reported. Decrease PCV observed in this study can be correlated with the decreased TEC. The hemoglobin value in the CD affected animals was found to be decreased (8.87 ± 0.52 g/dl) compared to the normal standard range (12-17.8 g/dl) which correlates with previous findings. The reduced platelets count (114.38 ± 9.26 x 103/µl) indicating thrombocytopenia occurs as a result of bone marrow depression by the virus. However, few workers observed platelets count within the normal range in CD affected animals. The mean corpuscular volume was found to be 54.17 ± 1.51fl. The mean corpuscular hemoglobin (20.66 ± 1.33pg) and mean corpuscular hemoglobin concentration (31.06 ± 1.06 g/dl).

The present study revealed that the TEC, PCV and hemoglobin values were considerably reduced, indicating that the disease produced microcytic hypochromic anaemia. The total leukocyte count was found (16.51 ± 1.34 × $10^3$/µl) within the normal range. However, different workers reported leukopaenia in CD affected animals. The absolute lymphocyte count recorded was 1.60 ± 1.26 ×$10^3$/µl which are

towards the lower value of the normal range. In contrast, many earlier workers have reported lymphocytopaenia in CD infected animals.

### 4.5 Clinical signs

Table 5 and 6 depicts the signs that 10 pups were showing i.e. of fever, inappetence, vomition, diarrhea, salivation, convulsion, dizziness, and posterior weakness. Four canine distemper positive pups were showing following clinical signs. Among four canine distemper positive dogs, all four (100%) dogs were showing signs of fever, profuse salivation, convulsion, posterior weakness; two pups (50%) were showing the signs of inappetence; one pup (25%) was showing the signs vomition and diarrhea.

**Table 5. Details of the sampling for detection of canine distemper virus through cPCR (Source: Teaching Veterinary Clinical Complex, OUAT)**

| Sl. No. | Breed | Sex | Age (in days) | Primary vaccination status | Clinical signs | cPCR Results for CDV |
|---|---|---|---|---|---|---|
| 1 | Desi | M | 35 | No | Fever, inappetence, profuse salivation, tremor, convulsions, posterior weakness | Negative |
| 2 | Shih Tzu | F | 70 | No | Fever, vomit ion, profuse salivation, tremor, convulsions, posterior weakness | Positive |
| 3 | Spitz | M | 44 | No | Fever, inappetence, profuse salivation, tremor, convulsions, posterior weakness | Positive |
| 4 | Labrador | M | 60 | No | Fever, diarrhea, profuse salivation, tremor, convulsions, posterior weakness | Positive |

**Table 6. Details of the sampling for detection of canine distemper virus through cPCR (Source: peripheral veterinary dispensary of Bhubaneswar city)**

| Sl. No. | Breed | Sex | Age (in days) | Primary vaccination status | Clinical signs | cPCR Results for CDV |
|---|---|---|---|---|---|---|
| 1 | Spitz | F | 40 | No | Fever, inappetence, profuse salivation, tremor, convulsions, posterior weakness | Negative |
| 2 | Bulldog | M | 48 | Yes | Fever, inappetence, profuse salivation, tremor, convulsions, posterior weakness | Positive |
| 3 | Dachshund | F | 33 | No | Fever, dizziness, profuse salivation, tremor, convulsions, posterior weakness | Negative |
| 4 | Desi | M | 50 | No | Fever, inappetence, profuse salivation, tremor, convulsions, posterior weakness | Negative |
| 5 | GSD | M | 42 | No | Fever, diarrhea, profuse salivation, tremor, convulsions, posterior weakness | Negative |
| 6 | Bulldog | M | 60 | No | Fever, vomit ion, profuse salivation, tremor, convulsions, posterior weakness | Negative |

**Table 7. Mean ± SE hematological parameters of four pups tested positive for CDV**

| Parameters | Mean ± S.E. |
|---|---|
| TEC ($10^6$/µl) | 4.57±0.23 |
| PCV (%) | 26.72±1.41 |
| Hb (g/dl) | 8.87±0.52 |
| THR ( $10^3$/µl ) | 114.38±9.26 |
| TLC ( $10^3$/ µl ) | 16.51±1.34 |
| Lymphocyte ( $10^3$/µl ) | 1.60±1.26 |

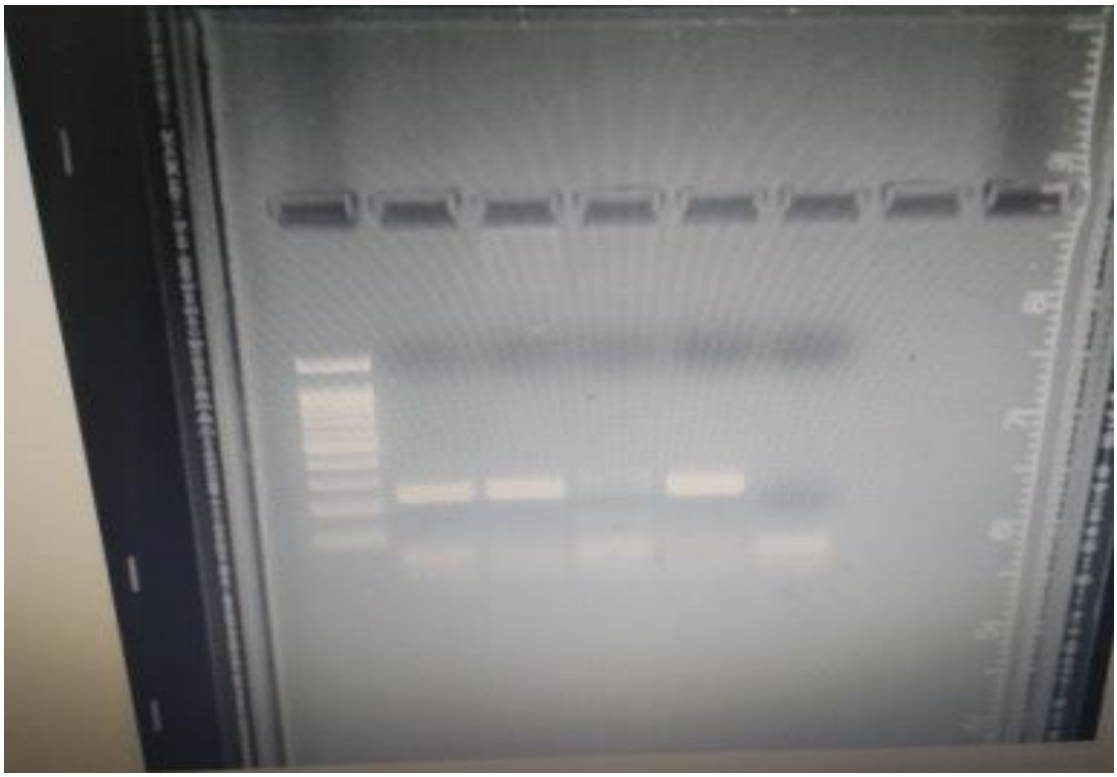


**Fig. 3: Visualization of PCR product**

# DISCUSSION

Canine distemper (CD), otherwise known as 'hard pad disease', is a highly contagious and potentially lethal viral disease of young growing canines. Animals inhabiting in wild like civet, raccoon, fox, palm civet, wolf, coyote, wild boar, skunk, ferret, and mink are also susceptible to distemper. Felidae, including many species of large cats as well as domestic cats are known to be capable of infection (Ikeda, 2001 and Greene and Appel, 2006). CD is caused by canine distemper virus (CDV). CDV is a RNA virus. CDV belongs to the genus *Morbillivirus* under family *Paramyxoviridae*. Genetic structure of CDV is closely resembled with the measles and rinderpest viruses. Virus gets entry into the host through ingestion, inhalation or transplacental routes and affects multiple systems i.e., gastro-intestinal, respiratory, integumentary, muscular and neurological systems (Namroodi *et al*., 2013 and Sykes, 2014). Dogs develop symptoms within a week after being infected. The disease is characterized by fever, nasal discharge, purulent ocular discharge, biphasic fever, inappetence, coughing, vomition, diarrhea and pustular dermatitis. In advanced cases virus attacks the central nervous system thereby resulting in symptoms like tilting of head, circling movement, partial or full paralysis, convulsive seizures, nystagmus, muscle twitching, convulsions and death. Canines that survive from CDV infection usually have permanent, irreparable nervous system damage.

Canine distemper is a vaccine-preventable disease along with the canine parvo virus, canine adenovirus, and rabies vaccines and covered under core vaccination program. The CDV in the surrounding environment can be destroyed by sunlight, heat and most disinfectants. But the risk is shedding of the virus from the distemper-infected dogs for up to several months, putting susceptible hosts around them at risk. Early and accurate diagnosis is of prime importance for appropriate therapeutic as well as preventive measures. Further, reports are available on incidence of CD in vaccinated dogs, possibly due to genetic variation of circulating field strains. Polymerase chain reaction (PCR) assay is the rapid, confirmatory gold standard diagnostic technique to detect and differentiate the disease within a short span of time and follow up studies thereof, hence present study is employed first time in Odisha.

In the present study two primers were used, one referred primer (Dung Van nguyen *et al.,* 2017) and another self designed primer. Referred primer is HF-

(AACTTAGGGCTCAGGTAGTC) 0.05 scale PAGE, HR-(AGATGGACCTCAGGGTATAG) 0.05 scale PAGE. We failed to record desired results using referred primer that tempted us to design a new primer targeting N gene (243 bp) of CDV. The designed primer is 1622 (F) -(ACCTGGGACCAGTGAAGAGA) HPSF 0.01 scale, 1864 (R) -(TCTCTCTGAGGGCTTTGAGG) HPSF 0.01 scale, respectively. Designed primer found more sensitive than referred primer for N genes of CDV as all the positive cases (n=4) were detected by former primer.

Of the 10 CD suspect pups included in the present study, presented either in the veterinary clinical complex of OUAT or peripheral veterinary dispensary of Bhubaneswar city, 04 pups tested positive to CDV through PCR. All four CD positive cases were between 48-70 days. Moller *et al*. (1993) described a rise in canine distemper (CD) in the urban dog population of the Copenhagen area. The cases were detected in canine from 10 weeks to 12 weeks of age. Kommonen *et al*. (1997) carried out a research work on outbreak of canine distemper in dogs having history of proper vaccination record in Finland. Of the 865 confirmed cases, 631 (73 %) were between 3 and 24 months of age. Jozwik and Frymus (2002) reported that pups in the age group of 3 to 6 months were more vulnerable to CDV infection. Lal *et al*. (2010) carried out a research work on incidence of canine distemper in pups. Out of 52 pups screened, only 10 pups were detected positive for canine distemper. The in general incidence of canine distemper amongst dogs was found to be 19.23 per cent. Age-wise incidence of canine distemper in dogs was the highest in pups up to 6 months of age (40%) followed by 12-18 months and above 36 months (20% each) and 6-12 months and 24-30 months (10% each) of age. The findings substantiated the fact that CD is a risk to all dogs, but dogs having no vaccination history and puppies under four months old are more susceptible.

The dawn of molecular tools and techniques brings diagnostic approach that is excellent with regard to sensitivity and specificity (Martella *et al*., 2008 and Soma *et al*., 2013). Pawar *et al*. (2011) isolated CDV from saliva and ocular secretions by targeting CDV N gene and concluded that saliva and conjunctiva swabs would be perfect samples for conventional PCR based confirmation of CDV genome. In the present study, saliva and conjunctiva swab samples were collected from CD suspected pups in ice pack and brought to the laboratory for analysis. Of the 6 samples

collected/received from the peripheral veterinary dispensary of Bhubaneswar city, only 1 sample tested positive and rest 5 samples were negative for CDV, though all 6 pups were showing typical signs of CD. This mismatch could be possibly due to improper collection and dispatch of biosamples resulting in CDV RNA denaturation.

Moller *et al*. (1993) screened unvaccinated dogs where all canine population was having unknown or obscure vaccination record. However, severe clinical cases were also screened in vaccinated canine population. Kommonen *et al.* (1997) carried out a study on outbreak of canine distemper in dogs having updated record of vaccination history in Finland. Out of the 865 confirmed cases, 631 (73%) were between 2 and 23 months of age, 487 of these had been vaccinated at least once and 351 (41 per cent) had a complete record of vaccination. Jozwik and Frymus (2002) reported that most (66%) of the affected dogs had no vaccination record against distemper, whereas (22%) of infected dogs were vaccinated at least once. In present study, among 4 CD positive cases, 3 were unvaccinated and 1 was vaccinated. It is inferred that CD has been reported both vaccinated and unvaccinated pups and this could be attributed to genetic variation between circulating field strain and vaccine strain.

In present study all CD positive dogs were found in descriptive breeds viz., Spitz, Labrador, Bulldog and Shih Tzu. Behera *et al.* (2014) conducted a study on ten dogs (7 crossbred, 2 Mongrels and 1 Doberman; 6 males and 4 females) of 2.5 to 7 months of age which were presented to the outpatient department of the College of Veterinary Science and Animal Husbandry; CAU, Mizoram with clinical signs of canine distemper. There was no sex or breed predilection. Dongre *et al*. (2013) studied the incidence of canine distemper infection in and around Mhow region of Madhya Pradesh. A total of 100 dogs were screened for incidence of CD infection, out of which 9 were found positive for canine distemper virus infection. Males were more prone for CDV infection than females. The sample size is not adequate enough to draw and definite conclusion with respect to sex and breed as reported by earlier investigators.

Namroodi *et al*. (2013) detected the CDV in rural canine population of Iran by conventional PCR and combination with Nested PCR and concluded the conventional PCR is sensitive and best appropriate method for diagnosis of CDV. Eha *et al*. (2014)

detected and quantified the viral RNA by real time PCR in Italy. Calderon *et al.* (2007) detected the CDV in both vaccinated and unvaccinated canines in Argentina. Simon- Martinez et *al.* (2008) identified the Canine distemper virus from serum samples by PCR in Mexican dogs and found that there was difference in N gene sequences obtained from vaccine and the circulating field strains and suggested that a novel CDV lineage might be present in Mexico. Pawar *et al*. (2011) tested the presence of CDV in ocular swabs and brain tissues by PCR assay. Fischer *et al*. (2013) detected and isolated the field and vaccine strains of CDV using reverse transcription followed by PCR assay in Brazil. They used the MSP-I restriction enzyme to differentiate the field and vaccine strains by restriction fragment length polymorphism (RFLP) analysis. Ashmi *et al*. (2017) detected CDV N gene by using conventional PCR and Nested PCR N gene positive samples were subjected for amplification of N and F genes. In present study conventional PCR was done using designed primer targeting N gene (243bp) of CD virus. PCR assay is not only gold standard test for CD diagnosis but also it is the rapid and confirmatory diagnostic assay.

Behera *et al*. (2014) conducted a research work to evaluate hematological parameters in 10 dogs with CDV infection. Hematology revealed marked (<0.05) reduction in the level of hemoglobin, RBC, hematocrit and thrombocytic count in 70 per cent of cases indicative of normocytic hypochromic anaemia as well as thrombocytopenia and neutrophilic leucocytosis with lymphopaenia in 80 per cent of cases. There was also occasional presence of intracytoplasmic inclusion bodies both in RBC and WBC in 20 per cent cases. Plasma biochemistry revealed mild hypoproteinemia and hyperglobulinemia with marked hypoalbuminemia. The total erythrocyte count (TEC), hemoglobin (Hb), packed cell volume (PCV), platelets count (THR) and mean corpuscular volume (MCV) was found lower than the standard range. Earlier workers opined that, long term presence of the virus in the bone marrow causes erythriod hypoplasia and hematological alterations. CDV infection releases interleukin-6 which causes sequestrating of iron into a low available form, thus iron is not available to the growing reticulocytes. Other possible causes of decrease TEC in canine distemper affected dogs as recorded in this study might be the production of inflammatory mediators, which could prevent erythropoesis and also shorten the life span of the RBC. Decrease PCV observed in this study can be correlated with the

decreased TEC. The hemoglobin value in the CD affected animals was found to be decreased (8.87 ± 0.52 g/dl) compared to the apparently healthy dogs. The reduced platelets count (114.38 ± 9.26 x $10^3$/µl) indicated thrombocytopenia as a result of bone marrow depression by the virus. However, few workers observed platelets count within the normal range in CD affected animals. Decreased PCV, MCH and MCHC were reported by earlier workers in CD positive cases (Kumar *et al*. 2011). The present study revealed that the TEC (4.57±0.23×$10^6$/µl), PCV (26.72±1.41%) and hemoglobin (8.87 ± 0.52 g/dl) values were considerably reduced, indicating that the disease produced microcytic hypochromic anaemia. The total leukocyte count was found (16.51 ± 1.34 × $10^3$/µl). However, earlier research workers found reduction in total leukocyte count in CD affected animals (Daga *et al*. 2013). The lymphocyte count recorded was 1.60 ± 1.26 ×$10^3$/µl which are towards the lower limit of the normal range. In contrast, many earlier workers have reported reduction in lymphocyte count in CD infected animals. This variation in hematological parameters could be linked with viral load, virulence of the antigen and immune power of the host.

Appel and Jones (1967) reported that CDV has been cultivated in canine alveolar macrophages. The attenuated form of CDV could readily be grown in epithelial and fibroblast cell lines but isolation of virulent CDV in these cells is difficult (Appel, 1978). But all these methods are time consuming and multiple blind passages are needed before any proper cytopathic effects of the virus been visible (Swati *et al*., 2016). In present study cell lines culture could not be attempted. However, cell culture study can be undertaken for in-depth study on genomics and proteomics. Whole genome sequencing of circulating field strains can be done so as to unveil the mutation, if any. This would help to enhance the efficacy of vaccine and minimize the vaccine failure.

# SUMMARY AND CONCLUSION

The present investigation on "**Molecular detection of canine distemper virus in clinically suspected dogs**" was carried out in the Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary Science and Animal Husbandry, Odisha University of Agriculture and Technology (OUAT), Bhubaneswar during the period from June 2019 to July 2020 with the objective of early detection of canine distemper virus (CDV) through molecular test.

A total of 10 clinically CD suspected pups presented either in the teaching veterinary clinical complex, OUAT or peripheral veterinary dispensary of Bhubaneswar city, were included in the study. All the pups except one were not vaccinated against CD. Clinical signs such as inappetence, fever, mucopurulent ocular and nasal discharge, vomition, respiratory distress, enteritis, seizure were common manifestations of the ailing pups. Both saliva (n=10) and ocular swabs (n=10) were collected in sterile phosphate buffer saline (1X PBS) and brought to the laboratory in ice-box.

Samples were processed for detection of viral nucleic acid by conventional PCR using N gene based designed diagnostic primers 1622 (F) - (ACCTGGGACCAGTGAAGAGA) HPSF 0.01 scale, 1864 (R) - (TCTCTCTGAGGGCTTTGAGG) HPSF 0.01 scale. Referred primer which is HF- (AACTTAGGGCTCAGGTAGTC) 0.05 scale PAGE, HR- (AGATGGACCTCAGGGTATAG) 0.05 scale PAGE could not able to detect CDV due to less sensitivity which tempted us to design a primer which is more sensitive. Designed primer could able to detect CDV prominently.

Out of 20 suspected biosamples (10 saliva swabs+10 ocular swabs) from 10 suspected pups, conventional PCR assay could able to amplify N gene of CDV with amplicon size of 243bp in 8 biosamples i.e., 4 saliva and 4 ocular swabs of 4 pups. On further Sanger's sequencing of the PCR products and nucleotide blast in NCBI GenBank showed 97.13 % sequence homology with that of the other CDV reference strains.

The hematological parameters such as TEC ($4.57\pm0.23\times10^6/\mu l$), PCV (26.72±1.41%), Hb (8.87 ± 0.52 g/dl), platelet count ($114.38 \pm 9.26 \times 10^3/\mu l$) were altered when compared with reference values and was indicative of microcytic hypochromic anaemia.

From the current study it can be concluded that:

- Proper collection of ocular and/or saliva samples from clinically CD suspected pups followed by conventional polymerase chain reaction (PCR) assay targeting N gene would be ideal laboratory procedure for confirmatory diagnosis of canine distemper (CD) within 6-8 hours of sampling.
- PCR assay, the gold standard test for rapid and confirmatory diagnostic technique of antigen detection, confirmed the circulation of canine distemper virus (CDV) among the susceptible canine population in this region.

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# APPENDIX

## BLAST analysis of N gene