SEROMONITORING AND DIAGNOSIS OF CANINE DISTEMPER

By

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THESIS

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DECLARATION

I hereby declare that this thesis entitled "**SEROMONITORING AND DIAGNOSIS OF CANINE DISTEMPER**" is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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We, the undersigned members of the Advisory Committee of **Sri. Kanagaraj, C.,** a candidate for the degree of **Master of Veterinary Science** in Preventive Medicine, agree that this thesis entitled "**SEROMONITORING AND DIAGNOSIS OF CANINE DISTEMPER**" may be submitted by Sri. Kanagaraj, C., in partial fulfilment of the requirement for the degree.

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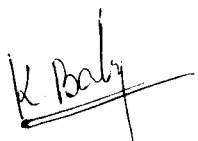

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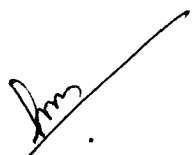

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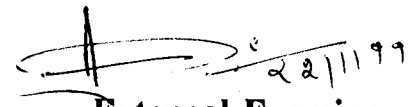

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KANAGARAJ, C.

Dedicated to KAU and my Guide

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LIST OF ABBREVIATIONS

AGID	-	Agar gel immuno diffusion
ASS	-	Ammonium sulphate solution
BSA	-	Bovine serum albumin
CD	-	Canine distemper
CDV	-	Canine distermper virus
CIEP	-	Counter immunoelectrophoresis
CSF	-	Cerebrospinal fluid
DAB	-	3/3-diaminobenzidine tetrahydrochloride
ELISA	-	Enzyme linked immunosorbent assay
FAT	-	Fluorescent antibody technique
HRPO	-	Horse radish peroxidase
IFT	-	Immuno fluorescent test
IPT	-	Immunoperoxidase test
mg	-	milligram
ml	-	millilitre
MLV	-	Modified live virus
OD	-	Optical density
OPD	-	O-phenylene diamine dihydrochloride
O.P. No.	-	Out patient number
PBS	-	Phosphate buffered saline
PBS-T	-	Phosphate buffered saline - Tween 20
PCV	-	Packed cell volume
SAS	-	Saturated ammonium sulphate
SNT	-	Serum neutralization test

Introduction

Chapter I

INTRODUCTION

Canine distemper (CD) is a severe, highly contagious, often fatal viral disease of dogs and certain other carnivores. It has world wide distribution. The virus nature of the disease was demonstrated in 1905 by Carre. Use of effective vaccines have reduced the incidence of canine distemper in domestic and captive wild carnivores. Certain wild species and unvaccinated carnivores continue to spread the virus and it makes the eradication virtually impossible (Appel and Carmichael, 1979).

The disease has been recognised for atleast two centuries and is endemic in most areas of the world. Epidemics have occurred in dogs in isolated areas where the disease had been absent for several years and a highly susceptible dog population had emerged (Bohm *et al.*, 1989). Similar epidemics have been described in wild life populations of raccoons, foxes and black-footed ferrets (Appel and Summers, 1995).

Canine distemper virus (CDV) is a widespread highly infective agent causing high morbidity and mortality in all breeds of dogs. The mortality rate is significantly contributed by secondary viral, bacterial and mycotic pathogens (Gorham, 1960; Appeland Gillespie, 1972).

It is an air borne disease and aerosol droplet infection from secretion of infected animal is the major source of infection. Though the virus is most abundant in respiratory exudates, it can be isolated from other body tissues and secretions including urine. Recovering dogs may shed the virus for several weeks, but not after they have fully recovered from the disease (Bhaumik, 1997).

Traditionally, the disease in dogs is characterized clinically by gastro-intestinal and respiratory symptoms, and at later stages with nervous complications manifested by myoclonus which usually ends fatally (Kirk, 1977).

An outbreak of canine distemper in captive large felids in North America (Appel *et al.*, 1994) and an epidemic in the lion population of the Serengeti National Park, Tanzania (Ke-Parker *et al.*, 1996) have demonstrated that the virus circulating in domestic and wild non-felid carnivores can cross species barriers into feline hosts (Harder *et al.*, 1991). Outbreaks among vaccinated dogs, however have been reported (Blixenkrone-Moller *et al.*, 1993). Outbreaks of distemper occurred in dogs throughout Europe during 1980 (Glardon and Stockli, 1985; Adelus-Neveu *et al.*, 1991) and in 1990, the disease reappeared in Finland after a break of 16

years. Before 1994, the infection occurred sporadically in Finland (Kommonen *et al.*, 1997).

Canine distemper is endemic in India and clinical cases occur throughout the year but the detailed epidemiological studies have not been worked out so far (Ganesan *et al.*, 1984). Outbreaks of canine distemper in dogs were also observed in Kerala (Saseendranath *et al.*, 1997).

The clinical diagnosis of canine distemper may be equivocal, particularly during the early catarrhal phase of the infection because of a great variability in range and severity of clinical signs. Therefore, a definitive diagnostic test for canine distemper is an important adjunct to the clinical diagnosis of this disease.

The present study was conducted with the following objectives for assessing the gravity of canine distemper in dog population by detecting the antibodies in serum and viral antigens in oculonasal discharge and corneal impression smear.

* Detection of canine distemper viral antigens using agar gel immunodiffusion test, counter-immuno electrophoresis and indirect immunoperoxidase test in oculonasal discharge and corneal impression smear, respectively.

* Detection of the canine distemper antibodies using agar gel immuno diffusion test, counter immuno electrophoresis and indirect ELISA in the sera collected from health and canine distemper suspected dogs.

* Assessment of CD antibody titre in healthy and CD suspected dogs by using indirect ELISA.

Review of Literature

Chapter II

REVIEW OF LITERATURE

Canine distemper is a highly contagious acute or subacute systemic viral disease which often has a high mortality rate in dogs and other carnivores (Kommonen *et al.*, 1997).

2.1 History

Distemper in dogs has been with us since the species became the domesticated companion of civilized man. The disease was well known to lay people as well as veterinarians, over a century ago (Blaine, 1817).

Dunkin and Laidlaw (1926) defined distemper as an acute fever with an incubation period of three to six days, and a diphasic temperature, coryza, vomiting, diarrhoea, cough, skin rash and an occasional corneal opacity and keratitis, from all of which many dogs might recover.

Green *et al.* (1930) demonstrated canine distemper virus in foxes and mink.

Koprowski *et al.* (1950) confirmed that the infectious agent responsible for epizootic canine encephalitis in the United States was the distemper virus.

Canine distemper was caused by a morbilli virus belonging to the family Paramyxoviridae, closely related to the measles virus of man and the rinderpest virus of ruminants, with predilection site for lymphoid, epithelial and nervous tissue (Appel and Gillespie, 1972).

The disease was first recognized during 1809 by Edward Jenner. However it was in 1905, that Carre identified the agent of canine distemper as a virus (Fenner *et al.*, 1993).

2.2 Epidemiology

2.2.1 Prevalence

2.2.1.1 Global

Canine distemper was reported to be one of the most prevalent viral infections in dogs in Belgium (Bjodtwedt *et al.*, 1969).

In the serosurvey of canine viruses in selected wild mammals, Jamison *et al.* (1973) confirmed canine distemper in an urban dog population and grey foxes in Sarasota Country, Florida.

In a study of respiratory diseases in 164 dogs, Binn *et al.* (1979) confirmed canine distemper using histopathology in 10 of 12 (83 per cent) dogs died. Sixty seven of 91 (74

per cent) dogs which arrived without CD antibody became ill and 16 of 67 (24 per cent) dogs with CD antibody had respiratory disease and only 3(4 per cent) died.

Geisel (1979) observed distemper infection in otters which developed clinical signs similar to CD and the infection was diagnosed by post mortem and histopathology.

Two Monte Carlo epidemics in conjunction with distemper epidemics in ferret populations were reported by Kelcker (1980).

Ramadass and Balu (1982) reported canine distemper in 68 (57.1 per cent) out of 119 dogs at the Madras Veterinary College Hospital with symptoms of gastroenteritis. Most of the cases were less than 6 months of age.

Stephenson *et al.* (1982) recorded the distemper infection in 4 wolves out of 57 in different areas of Alaska.

Asztalos *et al.* (1983) reported distemper outbreak in an colony of zoo mink in Hungary and the mortality was limited to 3.8 percent of 3120 animals after culling the severely ill animals.

Distemper outbreak was recorded in 1981 in Hungary in a unvaccinated stock of 469 standard black mink and 526 pastel breeding animals and 1323 younger mink showing typical

respiratory and central nervous signs with hyperkeratosis of snout and foot pads (Kovacs *et al.*, 1983).

Palmer *et al.* (1983) reported distemper outbreak in 22 beech martens in Switzerland during 1979-80 and it was confirmed as distemper virus by immunofluorescence test.

Zhang *et al.* (1983) recorded the distemper infection in two giant pandas and two pandas in Nanjiing Zoo, China during the winter in 1982.

An outbreak of canine distemper, involving atleast 23 free ranging wild raccoons, occurred at the Metropolitan Toronto Zoo between May and August 1981. This local outbreak was part of a major eruption of a disease in raccoons in Southern Ontario which began in early 1981 and persisted till mid 1983 (Cranfield *et al.*, 1984).

Bayoumi *et al.* (1985) reported canine distemper in sixteen of 43 stray dogs in Assiut Governorate, Egypt.

In a serosurvey of canine distemper in Nigeria, Ogunkoya *et al.* (1985) confirmed canine distemper in 108 (47 per cent) out of 232 apparently healthy indigenous dogs by neutralization test.

Steinhagen and Nebel (1985) reported distemper infection in 13 martens of 68 during 1979-84 in Schlaswig-Holstein.

Guo *et al.* (1986) reported canine distemper in 128 (56 per cent) coyotes out of 228 serum samples in Texas during 1975 to 1984.

Osterhaus *et al.* (1989) recorded canine distemper in one of three Baikal seals by confirming it with typical histopathological lesions and CDV neutralizing antibodies.

Grachev *et al.* (1989) reported canine distemper virus infection in several thousands of Lake Baikal seals during 1987 and 55 per cent of serum samples had 1:16 to 1:64 canine distemper virus neutralizing antibodies in neutralization test; 50 per cent were positive in the radioimmunoassay and 80 per cent were positive by ELISA.

Pop *et al.* (1989) reported that the incidence of canine distemper in Romania was 34 per cent in young puppies of 2.5-12 months of age and 1.5 per cent in dogs over 5 years of age.

In a serosurvey among 110 coyotes from 1989 to 1993, the prevalence of canine distemper antibodies was associated with the age of coyote with 88, 54, 23 and 0 per cent prevalence among adults, yearlings, old pups and young pups, respectively. Prevalence of CDV antibodies declined over time from 100 per cent in 1989 to 33 per cent in 1992 (Gese *et al.*, 1991).

During the study of health status of 220 dogs in Southern Africa, the highest point prevalence rate of CD was 5 per cent (Rautenbach *et al.*, 1991).

In a serological survey of 190 dogs in Liverpool, UK, from April 1985 to January 1986, 84 per cent of dogs were seropositive to CDV (Tennant *et al.*, 1991).

In a retrospective survey of 339 raccoons with canine distemper infection during 1985-90, an outbreak occurred in South Island and South Carolina (Hamir *et al.*, 1992).

Lyons *et al.* (1993) recorded morbillivirus infection in grey seal at the University of Guelph and it was confirmed as canine distemper virus.

Machida *et al.* (1993) reported canine distemper in a large number of free ranging raccoon dogs from September to December 1991 and confirmed it by post-mortem lesions, histopathology and immunocytochemistry.

Roscoe (1993) reported seventeen epidemics of canine distemper involving at least 615 raccoons between September 1977 and March 1991 in New Jersey and the epidemics occurred three times at four years interval in three areas and the prevalence occurred at the end of the mating season in March.

Thomas *et al.* (1993) reported distemper infection in two giant pandas and two pandas in Nanjiing zoo, China during the winter of 1982.

Alexander *et al.* (1994) in a serological survey of selected canine pathogens among free ranging jackals confirmed canine distemper in 4 (7.2 per cent) of 55 jackals.

Appel *et al.* (1994) recorded canine distemper in captive leopards, tigers, lions and a jaguar in 1991 and 1992 and the virus was identified by isolation technique.

Tipold *et al.* (1994) in a clinical study, confirmed the inflammatory form of distemper (encephalomyelitis) in 46 dogs and the non-inflammatory form in 38 dogs by histopathological examination and detection of viral antigen by IFT.

Johnson *et al.* (1995) conducted a survey of 613 private veterinary practices in Indiana to determine the prevalence of canine distemper during 1991 and 1992. The number of canine distemper cases in dogs were 973 and 1481 during 1991 and 1992 respectively.

Moll *et al.* (1995) recorded the distemper infection in 146 mustelids (132 martens, 5 badgers, 5 pole cats and 4 weasles) and 90 foxes in Southwest Germany over a two year period between 1989 and 1991.

Alexander *et al.* (1996) confirmed canine distemper in 10 free ranging African wild dogs in Chobe National Park, Botswana during 1994 by histopathological lesions and immunocytochemistry and concluded that the domestic dog population might constitute a significant disease threat to sympatric endangered wild dogs.

Gemma *et al.* (1996) confirmed canine distemper in 62 affected dogs in an outbreak using IPT.

Haas *et al.* (1996) reported the distemper infection in spotted hyaenas showing clinical signs similar to canine distemper. The closest homology (>99 per cent) was to a recently described CDV which caused high mortality in sympatric lions.

The blood samples from three wolves, two jackals and seven feral dogs were received and antibodies to canine distemper were detected in two wolves and four feral dogs by using ELISA. The positive result of sera samples might be due to the carrier status of infection or due to post infection status (John *et al.*, 1996).

Ke-Parker *et al.* (1996) observed that an epidemic caused by a morbillivirus closely related to canine distemper virus emerged abruptly in the lion population of Serengeti National

Park, Tanzania in early 1994 and by August 1994. Eighty five per cent of Serengeti lion population had anti CDV antibodies.

Henrisken *et al.* (1997) reported sporadic outbreaks of distemper in fur farms in Denmark (2-3 a year from 1990) and they also reported distemper in dogs throughout Denmark, with 4-36 cases occurring annually.

In an epidemiological survey among 152 coyotes, the prevalence rate of canine distemper was 37 per cent and it varied significantly from 1985 to 1990 (Cypher *et al.*, 1998).

2.2.1.2 India

Eventhough canine distemper was occurring in India for so many years, detailed epidemiological studies have not been worked out so far. During 1982 to 1983, an outbreak of canine distemper occurred in 373 (74 percent) out of 504 clinically suspected dogs and in 25 (38.46 percent) of 65 apparently healthy dogs (Ganesan *et al.*, 1984).

2.2.1.3 Kerala

A severe outbreak of canine distemper had occurred among dogs in Kerala during 1997. Dogs above six months of age were affected most severely and mortality ranged upto 80 per cent (Saseendranath *et al.*, 1997).

2.2.2 Host range

Many workers have reported canine distemper as one of the most important viral infections in dogs (Bjodtwedt *et al.*, 1969; Ganesan *et al.*, 1985; Murray, 1985; Pop *et al.*, 1989; Adelus-Neveu *et al.*, 1991; Harder *et al.*, 1991; Blixenkrone-Moller *et al.*, 1993; Machida, 1993; Lopez-Pehna *et al.*, 1994; Johnson *et al.*, 1995; Patronek *et al.*, 1995; Sihvonen *et al.*, 1995; Gemma *et al.*, 1996; Kommonen *et al.*, 1997 and Saseendranath *et al.*, 1997).

Distemper infection was also reported in raccoon dogs (Cranfield *et al.*, 1984; Hamir *et al.*, 1992; Roscoe, 1993), ferrets (Kelcker, 1980 and Lopez-Pehna *et al.*, 1994) wolves (Stephenson *et al.*, 1982), foxes (Moll *et al.*, 1995), martens (Palmer *et al.*, 1983 and Steinhagen and Nebel, 1985), Pandas (Itakura *et al.*, 1979 and Zhang *et al.*, 1983), hyaenas (Alexander *et al.*, 1995), japanese monkey (Yoshikwa *et al.*, 1989), seals (Grachev *et al.*, 1989), Coyotes (Cypher *et al.*, 1998), cats and pigs (Appel *et al.*, 1974) and other wild carnivores like leopards, tigers and lions (Appel *et al.*, 1994).

2.2.3 Transmission

Some workers (Krakowka *et al.*, 1977; Higgins *et al.*, 1981) reported that the neurological signs in puppies of 4-6

weeks of age, abortion, still birth or birth of weak puppies occurred due to transplacental transmission and enamel hypoplasia occurred due to neonatal infection with CDV.

Canine distemper virus is commonly spread by aerosol exposure and it is excreted from other body tissues and secretions, including urine upto 60-90 days. Contact between recently infected animals maintain the virus in a population, and a constant supply of puppies helps to provide a susceptible population for infection (Greene, 1990).

Bhaumik (1997) stated that aerosol droplet infection from secretions of infected animals is the major source of infection and the recovering dogs may shed the virus for several weeks but not after they have fully recovered from the disease.

2.2.4 Age

Some workers reported higher incidence of canine distemper in aged dogs, attributing it as old dog encephalitis or subacute encephalitis (Cordy, 1942, Wright, 1973; Imagwa *et al.*, 1980).

Hofman (1949) recorded canine distemper in dogs of 4-6 months of age.

Reinhard *et al.* (1955) reported the incidence of canine distemper being higher in young pups, however opined that susceptible dogs of all ages may become infected.

Appel and Carmichael (1979) observed that young dogs become susceptible to distemper when they lose maternal antibody, usually between 6-12 weeks of age and are therefore affected most often by the disease. The incidence of canine distemper decreased with age.

Ductalle *et al.* (1981) confirmed concurrent parvovirus and canine distemper infection in a 3 month old puppy by using immunoperoxidase test.

Of 119 dogs tested by AGID and IFT, 68 (57.1 per cent) were positive for canine distemper. Most of the cases were dogs less than 6 months of age (Ramdass and Balu, 1982).

Ganesan *et al.* (1985) observed that the incidence rate of canine distemper was high in old dogs due to their repeated exposure to infective surroundings and also due to lack of immunization. Incidence among animals below 6 months of age was relatively less.

In a serosurvey, Ogunkoya *et al.* (1985) reported CD in Nigerian dogs. Most of the positive results were in dogs less than 6 months or more than 3 years old.

In an epidemiological survey, Pop *et al.* (1989) observed that the incidence of CD was more in young animals of 2.5-12 months of age and was less in dogs over 5 years old.

In a serological survey, Tennant *et al.* (1991) confirmed canine distemper infection in 84 per cent out of 190 serum samples of dogs in a liverpool hospital population. The prevalence and antibody titres appeared to be influenced by age.

Malik *et al.* (1995) observed typical canine distemper in dogs of 8-17 weeks of age.

Saseendranath *et al.* (1997) reported a severe outbreak of canine distemper in dogs in Kerala and mortality ranged upto 80 per cent. Affected dogs were mostly above six months of age.

2.2.5 Sex

Lounela *et al.* (1997) reported 566 cases of distemper in dogs in 1994. There was no breed disposition, although mixed breed dogs were the largest group affected. More males than females were affected.

In the survival analysis of dogs, Morales *et al.* (1997) used kaplein Meier product limit method to determine survival in dogs after diagnosis of distemper. After analysing the 535

clinical cases, the log-range test showed no differences in survival with respect to age, sex, breed or season.

2.2.6 Breed

Canine distemper is widespread and is capable of affecting all breeds of dogs (Noon *et al.*, 1980).

Ganesan *et al.* (1985) reported canine distemper in different breeds Pomeranian, Alsatian, Dachshund, Doberman non-descript and cross breeds and other breeds like Rajapalayam, Labrador, Terrier etc. Among these breeds non-descript and crossbred dogs were mostly affected.

Greene (1990) found that canine distemper occurred in greyhounds, siberian huskies, Weimerinars Samoyeds and Alaskan malamutes.

In an experimental study, Baumgartner *et al.* (1995) observed that affected animals belonged mainly to the large breeds.

Patronek *et al.* (1995) reported in an epidemiological survey, that the risk of disease for pure bred dogs was 85 per cent lower than the risk of disease for mixed breed dogs.

Lounela *et al.* (1997) confirmed 566 cases of canine distemper in Finland in 1994 and stated that there was no

breed disposition, although mixed breeds were the largest group affected.

Outbreak of canine distemper in dogs in Kerala occurred during 1997. All the breeds viz., Alsatian, Doberman, Great Dane, Labrador, Rottweiler, Pomeranian and local breeds were affected (Saseendranath *et al.*, 1997).

2.2.7 Season

There was no seasonal variation in the occurrence of canine distemper among dog populations in temperate climate (Erno and Moller, 1961; Appel and Carmichael, 1979).

Anonymous (1963) observed that the incidence of canine distemper was high at the onset of inclement weather in the fall.

Canine distemper occurred throughout the year in dogs but the clinical disease manifested itself more frequently during the cold season of the year, when environmental conditions predispose the animal to the disease or lowers the resistance of the animal (Appel and Gillespie, 1972).

The incidence of canine distemper increased during July, August and January. Two peaks of incidence occurred, one in July and August and another in January. This could be due to

the low atmospheric temperature, as high atmospheric temperature inactivates the virus (Ganesan *et al.*, 1984).

In an epizootoliogical study of canine distemper in raccoon dogs, Roscoe (1993) reported that the peak period prevalence of canine distemper cases occurred at the end of the mating season in March.

Moll *et al.* (1995) reported canine distemper in mustelids and foxes. In that report, an increased number of distemper infection among mustelids was noted in summer.

2.2.8 Canine distemper vaccine failure

Joubert and Chappuis (1972) reported a outbreak of canine distemper in dogs which had been given one dose of combined distemper-hepatitis vaccine previously.

Bush *et al.* (1976) recorded distemper infection in Lesser panda, 2 weeks after vaccination with modified live virus vaccine and the disease clinically resembled canine distemper and was confirmed pathologically and by virus isolation.

Carpenter *et al.* (1976) reported distemper infection in four black footed ferrets in South Dakota, which died within 21 days after vaccination. The disease was confirmed by IFT, European ferret inoculation and SNT.

Itakura *et al.* (1979) found that Lesser pandas developed clinical signs similar to canine distemper and died 14-20 days after vaccination.

Distemper infection was reported in two young kinkajous following vaccination with MLV vaccine. The signs developed were diarrhoea and central nervous system signs and was confirmed by FAT (Kazacos *et al.*, 1981).

Glardon and Stockli (1985) reported an epidemic in the second half of 1984 and beginning of 1985, in Switzerland in 280 dogs and 71 per cent of cases had been vaccinated correctly.

Serious outbreaks of both canine distemper and canine parvo viral enteritis involving apparent vaccine failures, were reported in dogs in North Queensland in 1981-82 (Murray, 1985).

Gouveia *et al.* (1987) diagnosed 6.1 per cent cases as canine distemper showing clinical signs out of 3193 canine cases. The percentage of animals that had distemper was relatively high (22.3 per cent) although these had been previously vaccinated once or more than once.

Adelus-Neveu *et al.* (1991) reported an epidemic of canine distemper during 1987-89 in dogs of 3-12 months of age and

over 5 years throughout France due to the low immune status of the dog population. At the onset of the epidemic only 20-25 per cent of dogs had been vaccinated.

An outbreak of canine distemper was reported in 28 of 47 dogs in dogs home in northern Germany due to vaccine failure and the disease was confirmed by virus isolation and serological methods like IFT and neutralization tests (Harder *et al.*, 1991).

Blixenkrone-Moller *et al.* (1993) reported a canine distemper outbreak in the urban dogs in Copenhagen area where the disease was primarily manifested in young dogs. They confirmed the disease in 49 out of 66 sera samples of clinically suspected dogs and in 27 out of 56 specimens of conjunctival cells by IFT.

An outbreak of canine distemper in pure bred pet dogs in Indiana during 1992-93 was reported. The risk of disease for pure bred dogs was 85 per cent lower than the risk of disease for mixed breed dogs (Patronek *et al.*, 1995).

Sihvonen *et al.* (1995) reported a canine distemper epidemic in dogs, from January to March 1995, in Finland. He confirmed 790 cases out of 2821 dogs. Among these 790 dogs, 4 per cent were unvaccinated, 22 per cent had an unknown

vaccination status and 74 per cent had been vaccinated atleast once.

Canine distemper reappeared in dogs in Finland in 1990 after a 16 year absence. In 1994 to 1995, an outbreak occurred in areas with a high density population which included dogs vaccinated against distemper (Kommonen *et al.*, 1997).

In an epidemiological study of canine distemper in Finland in 1994, Louenla *et al.* (1997) reported 566 cases of distemper in dogs. The number of cases increased from July to October and then declined in which 64 per cent of the affected dogs had been properly vaccinated.

2.3 Clinical signs

Bjodtvedt *et al.* (1969) and Wright *et al.* (1974)had reported that pneumonia occurred with the systemic disease in canine distemper. Yet recently the virus has been considered for causing respiratory disturbances without enteritis, conjunctivitis or nervous symptoms.

Fairchild *et al.* (1971) reported canine distemper in dog was characterized by a great variability in range and severity of clinical signs.

Gorden (1971) observed that nervous signs occurred in atleast 50 per cent clinical cases of canine distemper and the symptoms were chorea, epilepsy, spastic or flaccid paralysis or psychosomatic disturbances such as depression or excitement depending upon the area of brain involved.

Canine distemper, the clinical disease arising from the canine distemper virus infection in susceptible hosts varied greatly depending upon the strain of virus involved, the virus dose at exposure, the suceptibility of the host species and the immune response of each of animal (Appel and Gillespie, 1972; Appel *et al.*, 1981 and Krakowka *et al.*, 1985).

Stevans and Osburn (1976) reported that the abortion, still birth or birth of weak puppies were noted depending upon the stage of gestation at which CDV infection occurred.

Kirk (1977) observed convulsive seizures, incoordination, pacing, circling and psychic changes in acute cases of canine distemper.

Gossett *et al.* (1982) noticed mucopurulent nasal discharge, cough, conjunctivitis and tremors in young dogs. Later the dog became moribund and died within 14 days of infection.

Ramadass and Balu *et al.* (1982) reported canine distemper in 68 (57.1 per cent) out of 119 dogs showing the symptom of gastroenteritis. The positive animals showed digestive complications than nervous involvement.

Ganesan *et al.* (1984) reported that canine distemper was clinically characterized by gastro-intestinal and respiratory symptoms and at later stages with nervous complications, manifested by symptoms ranging from mild inapparent symptoms to severe gastro intestinal and neurological symptoms.

Higgins *et al.* (1988) reported that neurological signs occurred in canine distemper and chronic relapsing neurologic deteroriation occurred with intermittent recovery and later acute episode of neurologic dysfunction in the same dog.

In a review of 71 cases of dog distemper, Pop *et al.* (1989) reported 50 per cent and 32 per cent of affected animals showing digestive and respiratory symptoms, respectively.

Greene (1990) stated that neurological signs varied according to the area of CNS involved. Hyperaesthesia and cervical rigidity developed as a result of meningeal inflammation. Seizures, cerbral and vestibular signs, paraparesis or tetraparesis with sensory ataxia and myoclonus were common. Chewing gum type of seizures occurred in dogs

developing polioencephalomalacia of the temporal lobes. Myoclonus was present in the absence of neurological signs. Due to spinal cord damage, there was upper motor neuron paresis of affected limb associated with myoclonus. The rhythmic contractions were present while the dog was awake or sleeping.

Cheng and Tham (1991) noticed pyrexia, ocular and respiratory signs suggestive of distemper and myoclonus of forelimbs and hindlimbs in dogs suffering from CD.

Nieto *et al.* (1992) observed progressive ataxia and paralysis of short duration in dogs immediately after vaccination.

Tipold *et al.* (1992) stated that canine distemper was a multisystemic disease often with severe neurological signs. Myoclonus was almost pathognomonic for this disease but this occurred in less than half of the cases. The prognosis of nervous distemper was generally poor although dog could recover from this disease.

Machida *et al.* (1993) reported canine distemper in raccoon dogs showing serous or mucopurulent oculonasal discharges, respiratory distress, vomiting, diarrhoea with mucus and blood and generalized scurfiness of skin.

Roscoe (1993) reported seventeen epidemics of canine distemper involving atleast 615 raccoons in New Jersey and the commonest clinical symptom was lethargy.

In an epizootic of canine distemper in kenya, signs observed in clinically ill domestic dogs were consistent with canine distemper and included listlessness, decreased appetite, bilateral serous to mucopurulent oculonasal discharges and diarrhoea and the mortality rate was 21 per cent, 50 per cent and 38 per cent in 1990, 1991 and 1992, respectively (Alexander *et al.*, 1994).

Alexander *et al.* (1996) reported canine distember in 10 free ranging African wild dogs showing the signs of emaciation and mucopurulent ocular discharge.

In an outbreak of canine distemper in Tokyo, 62 dogs were affected with canine distemper. Thirty four dogs had respiratory and gastrointestinal signs associated with CNS signs and 28 had CNS signs only (Gemma *et al.*, 1996).

Ke-Parker *et al.* (1996) recorded distemper in lions showing grandmal seizures and myoclonus. The animals that died had encephalitis and pneumonia.

Of 31 dogs, in which distemper had been confirmed by demonstration of inclusion bodies, 23 had shown respiratory

signs, 15 nervous signs, 10 diarrhoea and 2 vomiting (Seong and Seo, 1996).

Silver *et al.* (1996) noticed seizures, facial twitching and rhythmic contractions of the tongue in 2.5-year-old male dog suffering from canine distemper.

Thirunavukkarasu *et al.* (1996) observed congested mucous membrane and ocular mucopurulent discharges with chorioretinal lesions in dogs suffering from CD and stated that ocular signs were frequently associated with distemper in dogs.

Henrisken *et al.* (1997) reported one sporadic outbreaks of distemper in dogs. Most of them showed ocular discharge, reduced appetite and fever.

Inomata *et al.* (1997) confirmed canine distemper in 158 (1.5 per cent) out of 10423 dogs. Most of the positive cases showed fever, weakness, anorexia, vomiting, diarrhoea, nasal discharge and conjunctivitis.

An outbreak of canine distemper occurred in dogs in Finland. The signs recorded were anorexia, conjunctivitis and fever that frequently fluctuated between 39.2°C and 40°C and in severe cases, respiratory illness. In the early stages of the infection, vomition and diarrhoea lasted for one to two days. In severe cases, the animals remained depressed and

anorectic for several weeks and showed serous nasal and ocular discharges which became mucopurulent. Some dogs exhibited a reddening of skin followed by a pustular rash. Hyperkeratosis of foot pads and nose and CNS signs were also observed. Mortality reached upto 30 per cent (Kommonen *et al.*, 1997).

Lounela *et al.* (1997) reported 566 cases of canine distemper in Finland in 1994. Most of the affected dogs showed typical signs except for more frequent dermatological signs.

An outbreak of canine distemper in dogs occurred in Kerala and affected dogs showed loss of appetite, vomition, respiratory distress, conjunctivitis with purulent lacrimal discharge, thick mucopurulent nasal discharge and vesicular and pustular dermatitis. Diarrhoea was seldom reported. The course of the disease was 14 days to 21 days. In later stages, most of the dogs were either showing inco-ordinated movement or paraplegia, leading to lateral recumbency and death. Hyperkeratosis of foot pad was also observed in some chorea cases. The surviving dogs were showing myoclonus and seizures. Mortality ranged upto 80 per cent (Saseendranath *et al.*, 1997).

Tudury *et al.* (1997) noticed postural reactions (87.65 per cent), decreased tear production (83.95 per cent), myoclonus (75.30 per cent), paresis (69.12 per cent),

conjunctivitis (56.79 per cent), and chorioretinitis/digital and nasal hyperkeratosis (51.85 per cent) in distemper infected dogs.

2.4 Diagnosis

Traditionally the canine distemper is characterized clinically by gastrointestinal and respiratory symptoms and at later stages with nervous complications manifested by myoclonus which usually ends in fatality. In the early stages of the infection, it is difficult to diagnose clinically (Kirk, 1977).

2.4.1 Laboratory diagnosis

Cutler and Averill (1969) reported that an increase in the protein (>25 mg/dl) and cell count (>10 cells/μl with a predominance of lymphocytes) were noticed in cerebrospinal fluid. These abnormalities were detectable in dogs with neurological signs.

Engen (1971) reported that the haematological changes due to canine distemper include decrease in PCV from 41.9 to 36.8 per cent. Serum concentration of sialic acid increased from 50.9 mg/100 ml to 79.7 mg/100 ml.

Stevans and Osburn (1976) observed decreased albumin and increased α and gamma globulin concentrations in neonates with distemper infection. Marked hypoglobulinemia has been found in puppies infected prenatally or neonatally with CDV due to persistent immunosuppression caused by the virus.

Gossett *et al.* (1982) observed non-generative anaemia (PCV 28 per cent), lymphopenia (100 cells/μl) and monocytosis (1800 cells/μl) in a clinical study of distemper in dogs.

Distemper inclusions were detected, on examination of stained peripheral blood smears, in low numbers, in circulating lymphocytes and with even less frequency in monocytes, neutrophils and erythrocytes (McLaughlin *et al.*, 1985).

In the cerebrospinal fluid of distemper infected dogs, increased anti CDV antibody was noticed (Johnson *et al.*, 1988; Potgieter and Ajidagba, 1989).

During studies on hematology of distemper infected dogs, Greene (1990) found an absolute lymphopenia which frequently persisted in young dogs with rapidly progressive systemic or neurological signs and thrombocytopenia as low as 30,000 cells/μl.

Tipold *et al.* (1994) studied mononuclear pleocytosis and intrathecal immunoglobulin synthesis in CSF of distemper infected dogs.

Tudury *et al.* (1997) noticed anaemia (48.05 per cent) lymphopenia (51.95 per cent) and mild changes in the CSF characterised by an increase in total protein (77.33 per cent) and lymphocytic pleocytosis (50.72 per cent) in 81 distemperoid encephalomyelitis affected dogs.

Abdate *et al.* (1998) observed that the dogs affected by canine distemper showed increased macrophages, presence of specific inclusion bodies and an increased total protein concentration and gamma globulin fraction in the cerebrospinal fluid.

2.4.2 Agar gel immunodiffusion (AGID) test

Of 119 dogs showing gastroenteritis, Ramadass and Balu (1982) confirmed canine distemper in 68 (57.1 per cent) cases by AGID.

Ganesan *et al.* (1984) studied the canine distemper infection in 373 (74 per cent) of 504 suspected dogs in conjunctival and nasal suspensions. He also screened 67 apparently healthy dogs and found out 28 (37.3 per cent)

having CD viral antigen in their conjunctival and nasal secretions.

2.4.3 Counter immunoelectrophoresis (CIEP)

Fifty six dogs were tested for canine distemper by agar gel immunodiffusion (AGID) test and counter immuno electrophoresis (CIEP) using lacrimal discharge. Forty four (78.57 per cent) dogs gave positive reaction with CIEP and 42 (75 per cent) dogs gave positive reaction with AGID. CIEP was found to be more sensitive than AGID (Ramadass *et al.*, 1983).

2.4.4 Giemsa staining

Viral inclusions had occasionally been reported in peripheral blood cells in clinical cases of CD, particularly in lymphocytes (Cello *et al.*, 1959; Ettinger *et al.*, 1975).

Ocampo (1972) reported that canine distemper could be confirmed by detecting intracytoplasmic inclusion bodies in the cells of corneal impression smear, which were not present in all cases.

Smith *et al.* (1972) stated that the finding of characteristic intracytoplasmic inclusion bodies in the cells of dogs with respiratory or nervous signs of distemper was the best method of confirmation of the diagnosis.

Some authors observed that inclusion bodies were seen, in the cytoplasm of lymphocytes from a dog which developed distemper (Watson and Wright, 1974; Smith *et al.*, 1983).

Schalm *et al.* (1975) and Schalm (1980) suggested that the inclusions were rare in peripheral leukocytes and more common in immature erythrocytes in dogs suffering from CD.

Dagle *et al.* (1979) observed that numerous esinophilic, round to oval cytoplasmic inclusions surrounded by a slight halo in the transitional epithelial cells were seen in the haematoxylin and eosin stained sections of urinary bladder of distemper infected dogs.

Gossett *et al.* (1982) observed intracytoplasmic inclusions in lymphocytes stained by using Wright's stain and they were blue-gray, oval or irregular in shape. He further demonstrated erythrocytic inclusions in similarly stained smears in CD.

Jones and Hunt (1983) stated that canine distemper viral inclusions were more frequently seen in neutrophils than in lymphocytes.

2.4.5 Indirect immunoperoxidase test

Ductalle *et al.* (1980) found that combined peroxidase and routine histologic staining using haemotoxylin and eosin for

the detection of inclusion bodies in epithelial cells and nerve cells was a useful method in the diagnosis of canine distemper.

Ductalle *et al.* (1981) reported that intracytoplasmic inclusion bodies could be detected by using IPT in tonsil, stomach, jejunum, colon and urinary bladder of distemper infected dogs.

Higgins *et al.* (1982) found that an indirect immunocytochemical labelling technique, using horse radish peroxidase-conjugated antibody was used to detect the intra cellular and the surface membrane localization of CDV antigen during productive virus replication in infected vero cells.

Miry *et al.* (1983) confirmed 14 cases of canine distemper out of 42 cases using IPT.

Sadoff *et al.* (1990) reported the presence of CDV antigen in the nuclei and cytoplasm of many astrocytes in distemper infected dogs by using immunoperoxidase test.

Ayround *et al.* (1992), by using immunoperoxidase staining technique, demonstrated the presence of intracytoplasmic inclusion bodies in pulmonary macrophages and lymphoid cells of ranch foxes suffering from canine distemper.

Nieto *et al.* (1992) developed an indirect immuno-peroxidase staining technique using a monoclonal antibody against the nucleocapsid of CDV for the demonstration of inclusion bodies which were seen in the epithelial and nervous tissue cells.

Gathumbi (1993) developed a peroxidase technique for the confirmation of suspected CDV infection in Kenya. The inclusion bodies were sharply stained and were seen in the astrocytes, microglia and ependymal cells in the brain and spinal cord, in the cells of the urinary bladder epithelium, in the gastrointestinal mucosa and also in respiratory epithelium.

Canine distemper antibodies were assayed by Soma *et al.* (1994) using immunoperoxidase technique and compared with neutralization methods and found that there was good agreement between the results of IPT and neutralization tests. They concluded that IPT was as sensitive as neutralization methods.

2.4.6 Indirect ELISA

Forghani and Schmidt (1979) stated that ELISA was an accepted immunologic tool for the detection of various classes of antibodies and specific antibodies against known antigen.

Noon *et al.* (1980) developed an ELISA for the detection of antibody against canine distemper and compared it with SNT. The sera of 273 random source adult dogs were examined and there was good agreement between the ELISA and SNT. They found that ELISA had a sensitivity of 95.7 per cent and specificity of 98 per cent.

Bernard *et al.* (1982) developed a highly specific and sensitive ELISA for the detection of canine IgG against CDV antigens and compared the ELISA results with SNT. Significant agreement between the ELISA and SNT was shown.

Valencia *et al.* (1987), in comparing the IFT and ELISA for the detection of antibody against canine distemper stated that the results of IFT and ELISA were of similar sensitivity.

Blixenkerone-Moller *et al.* (1993), confirmed the canine distemper infection in 49 out of 66 samples by using IgM ELISA and compared it with immunofluorescence. By using IFT, 27 out of 56 specimens of conjunctival cells were detected to contain the CDV antigen.

Gemma *et al.* (1995) by using the ELISA for the detection of antibodies against CDV and comparing the results with VNT suggested that ELISA was a rapid and reliable method for the serological survey of CDV infection.

John *et al.* (1996) confirmed canine distemper infection in two wolves and four feral dogs by ELISA and the positive result of sera samples might be due to the carrier, status of infection or due to past infection.

Materials and Methods

Chapter III

MATERIALS AND METHODS

The study was carried out in the Department of Preventive Medicine, College of Veterinary and Animal Sciences, Mannuthy during July 1997 - June 1998.

3.1 MATERIALS

3.1.1 Glasswares and reagents

In this study, Borosil brand of glassware, Laxbro plastics and analytical or guaranteed reagent grade of chemicals were used.

The materials were processed using standard methods (Hoskins, 1967) and sterilized either in hot air oven or autoclave depending upon the material to be sterilized.

3.1.2 Collection of samples

The corneal impression smears and oculonasal discharge were taken from 113 canine distemper suspected dogs. CDV antigens were detected in corneal impression smears subjected to Giemsa staining and indirect immunoperoxidase test it was also detected in oculonasal discharge by using AGID and CIEP.

Blood from three hundred dogs of various ages from different localities of Thrissur were collected. Serum was separated and stored at -20°C. The serum was screened for the presence of antibodies against canine distemper by serological tests AGID, CIEP and ELISA.

The smears were fixed in methanol for 15 min and then air dried. Conjunctival and nasal discharges were collected in a sterile test tube containing one millilitre of 0.85 per cent sodium chloride from 100 suspected canine distemper cases for the detection of CD viral antigen by AGID and CIEP.

Detailed history of each case was collected in the format (Appendix I).

3.1.3 Agar gel immunodiffusion (AGID) test

Reagents

Noble agar (DIFCO)	-	1.2 g
Normal saline to make	-	100 ml

Staining

Amido black (1 per cent)

Glacial acetic acid (7 per cent)

Hypertonic saline (1.5 per cent)

Counter immunoelectrophoresis (CIEP)

Reagents

Agarose	-	1.2 gm
Veronal acetate buffer	-	100 ml

Veronal acetate buffer (pH 8.6)

Barbitone sodium	-	10.31 gm
Barbituric acid	-	1.84 gm
Sodium acetate	-	6.8 gm
Sodium azide	-	0.5 gm
Distilled water to make	-	1000 ml

3.1.4 GIEMSA staining

Stock solution

Giemsa dry stain	-	7.36 gm
Glycerol	-	500 ml
Methanol	-	500 ml

Working solution

Stock giemsa solution	-	1 ml
Phosphate buffer solution (pH-7.0)	-	19 ml

3.1.5 Globulin separation

Saturated ammonium sulphate solution (SAS)

Ammonium sulphate	-	760 gm
Distilled water	-	1000 ml

Working ammonium sulphate solution (66 per cent)

Saturated ammonium sulphate solution	-	66 ml
Distilled water	-	34 ml

Ammonium hydroxide solution

Normal saline

10 per cent Barium chloride solution

Borate buffered saline pH 8.5

Five parts of borate buffer was added to ninety five parts of normal saline.

Borate buffer

Boric acid	-	6.184 gm
Borax	-	9.536 gm
Sodium chloride	-	4.834 gm
Distilled water	-	1000 ml

The reagents used for preparing borate buffer saline were added to one litre volumetric flask containing 600-800 millilitre of distilled water and shaken until contents were

completely dissolved. To this, distilled water was added to make the volume to one litre and the pH was adjusted to 8.5.

3.1.6 Conjugation

0.1 M potassium phosphate solution

Potassium phosphate	-	1.36 g
Distilled water	-	100 ml

1 per cent gluteraldehyde solution

Gluteraldehyde (25 per cent solution)	-	0.2 ml
Distilled water	-	9.8 ml

Total protein and albumin estimation

Reagent 1	Biuret reagent
Reagent 2	Buffered dye reagent
Reagent 3	Protein standard

3.1.7 Immuno electrophorosis

Materials

Agar	:	1.2 gm
Veronal acetate buffer (pH 8.6)	:	100 ml

3.1.8 Indirect immunoperoxidase test

Tris-Hcl (pH 7.6)

A : 0.2 M (Tris 2.42 g/100 ml)

B : 0.2 N Hcl

50 ml of A + 38.4 ml of B

Distilled water to make - 200 ml

Tris buffer (pH 7.2)

Tris - 0.605 gm

Distilled water to make - 100 µl

pH was adjusted to 7.2 with 0.1 per cent sodium hydroxide.

Peroxidase Conjugated Chicken Anti Rabbit Immunoglobulin (1:500) solution (Sigma, USA)

Peroxidase - conjugated chicken antirabbit immunoglobulin - 1 µl

PBS - 5 ml

Substrate solution

3'3 - Diamino benzidine tetrahydrochloride - 5 mg

Tris-Hcl buffer - 10 ml

Hydrogen peroxide - 30 µl

3.1.9 Indirect ELISA

Canine distemper virus

Live attenuated embryo adapted vaccine, obtained from IVPM, Ranipet was used as antigen.

ELISA plates

96 well Flat bottom plates from Tarson

Bovine serum albumin (BSA)

BSA - 2 g

PBST - 100 ml

Phosphate buffered saline (PBS) (pH-7.2)

PBS stock (10x)

Sodium chloride	- 80 gm
Potassium chloride	- 2 gm
Disodium hydrogen phosphate	- 11.33 gm
Potassium dihydrogen phosphate	- 2 g
Distilled water to make	- 1000 ml

pH was adjusted to 7.2

PBS working solution (pH 7.2)

PBS (10x)	- 100 ml
Distilled water to make	- 1000 ml

PBS Tween 20 (PBS-T)

Tween 20	-	0.5 ml
PBS (10x) solution	-	100 ml
Distilled water to make	-	1000 ml

Carbonate-bicarbonate buffer (pH 9.6)

Sodium carbonate	-	1.59 gm
Sodium bicarbonate	-	2.98 gm
Distilled water to make	-	1000 ml

pH was adjusted to 9.6

Phosphate-citrate buffer (pH 5.0)

0.2 M dibasic sodium phosphate	-	25.7 ml
0.1 M citric acid	-	24.3 ml

pH was adjusted to 5.0

Peroxidase Conjugated Rabbit Anti-dog Immunoglobulin (1:10) solution

Peroxidase - conjugated rabbit anti-dog immunoglobulin	-	1 μl
PBST	-	0.09 ml

Peroxidase Conjugated Rabbit Anti-dog Immunoglobulin (1:15000) solution (Sigma, USA)

Peroxidase - conjugated rabbit anti-dog immunoglobulin	-	1 μl
PBST	-	14.99 ml

Substrate solution

OPD (O-phenylene diamine dihydrochloride, sigma)	-	30 mg
Phosphate-citrate buffer	-	75 ml
Hydrogen peroxide (30 per cent)	-	30 µl

3.2 METHODS

3.2.1 Preparation of antidog whole serum

The animal was immunized by the following schedule.

One millilitre of whole dog serum was homogenized with one millilitre of Freund's complete adjuvant and was given intramuscularly to a healthy rabbit aged 6 months. Three booster doses of one millilitre whole dog serum without adjuvant were given at weekly interval by same route.

Ten days following the last injection test bleeding was done to assess the antibody response by AGID and immunoelectrophoresis. When the results were found satisfactory, the animal was bled, the serum separated, inactivated at 56°C for 30 min and stored at -20°C in a small aliquotes of one millilitre each to be used as rabbit antidog whole serum.

3.2.2 Preparation of canine distemper (CD) hyper immune serum

Snyder Hill strain of canine distemper virus vaccine obtained from Institute of Veterinary Preventive Medicine, Ranipet was used to produce CD hyperimmune serum in rabbits.

Two male rabbits aged six months used for this purpose.

Totally four injections were given at interval of 10 days. Freund's complete adjuvant was used for the first injection and Freund's incomplete adjuvant was used for subsequent injections. Seven days after the last injection, the rabbits were bled and tested by AGID for the antibody. When the results were found satisfactory, the rabbits were bled and serum was separated, inactivated at 56°C for 30 minutes and stored in small aliquotes of 2 millilitre each at -20°C.

3.2.3 Agar gel immunodiffusion test (AGID)

The test was carried out as per the method described by Carpenter (1965).

3.2.3.1 Staining and preservation

The slide was kept in 1.5 percent hypertonic saline solution with three changes at 8 hrs interval. The slides

were dried by clamping them between the moist filter paper for overnight at 37°C. When the slides were dried, they were immersed in one per cent Amidoblack solution for three to five minutes. Excess stains were removed by keeping the slides in seven per cent aquous glacial acetic acid till the background gel turns optimally clear. The slides were dried and kept it in a polythene bag after labelling.

A white precipitate between the antigen and suspected serum sample was taken as positive.

3.2.4 Counter immunoelectrophoresis

This test was carried out as per the method described by Ramadass *et al.* (1983).

3.2.4.1 Staining and preservation

Staining was carried out as described in AGID.

A white precipitate between anodal and cathodal wells was taken as positive.

3.2.5 Immunoelectrophoresis

The test was carried out as per the method of Williams and Chase (1971) with some modifications.

Agar (1.2 per cent) in veronal buffer was melted and three millilitre of melted agar at 50°C was poured onto each slide kept on a levelled surface. The agar was allowed to solidify initially at room temperature and subsequently at 4°C. Wells and troughs in between the wells were cut on each slide.

After removing the agar, the wells were filled with antigens. A drop of bromophenol blue dye was added to the side of the well as an indicator. The slides were then placed in the electrophoresis chamber in such a way that the antigen wells were nearer to the cathode than to anode. Contact between the slides and the buffer was effected by filter paper wicks on each end of the slide. Power supply at the rate of 5 mA per slide was given and the electrophoresis was continued till the indicator dye reached one centimetre away from anode end of the slide.

The power supply was disconnected, slides were taken and the agar in the troughs were removed carefully. The troughs were then filled with respective antisera (antidog whole serum) and left at room temperature in electrophoretic chamber for 20-24 hrs.

The slides were examined against a light for the development of precipitin arcs and then the slides were washed and stained as for AGID.

3.2.6 Giemsa staining

The staining procedure was carried out as per the method described by Bancroft (1984).

3.2.7 Globulin separation

Globulin from the normal dog serum was separated using the procedure described by Garvey (1977).

Fifty millilitre of 66 per cent ammonium sulphate solution (ASS) was added dropwise to a fifty millilitre of serum sample while stirring. The stirring of serum ASS mixture was continued for 30 min after the addition of last drop of ASS and precipitate was allowed to stand overnight at 4°C. Next day the suspension was dissolved in enough saline to restore the original volume of serum and reprecipitated twice following the above procedure, omitting overnight keeping of suspension at 4°C. The precipitate from the third precipitation was dissolved in borate buffered saline to a final volume of 20 ml. The ammonium sulphate was removed from the precipitate by dialysing against borate buffered saline at 4°C.

3.2.7.1 Estimation of globulins

Globulin was estimated following the qualigens kit. Serum globulin was calculated by substracting the serum

albumin value from the total serum protein (Benjamine, 1985). The results were recorded in gm/dl.

3.2.7.2 Preparation of antidog globulin

Antidog globulin was raised in two rabbits by dissolving the serum globulins in borate buffered saline having a concentration of 15 mg/ml. Seven days following the last injection rabbits were bled, serum separated inactivated at 56°C for 30 min and tested by AGID and immunoelectrophoresis. When the results were satisfactory, globulins were used for HRPO conjugation after checking its purity and concentration.

3.2.7.3 Conjugation

The labelling of antidog globulin with horse radish peroxidase was done as per the procedure described by Avrameas (1969) with slight modifications.

The antidog globulin was reconstituted with borate buffer to obtain 7 mg of globulin/ml and the pH was adjusted to 6.9 by addition of 0.1 M solution of potassium phosphate. For each ml of above solution, 12 mg of HRPO enzyme was added and after its complete dissolution, 0.05 millilitre of gluteraldehyde was added. The mixture was shaken for 2 h at room temperature by end-over-end rotation. The product thus obtained was then dialysed overnight at 4°C against

physiological saline, pH 7.4. Next day the solution was centrifuged for 15 min at 1500-2000 g. The supernatant was collected and stored in small aliquotes at -20°C.

3.2.8 Indirect immunoperoxidase test

The test was carried out as per the method described by Ductalle *et al.* (1980) with slight modifications.

The corneal impression smears were fixed in methanol for 15 min and the smears were treated with canine distemper hyperimmune serum (1:10) raised in rabbits incubated at 37°C for 45 min in a humid chamber.

The slides were then washed with Tris buffer twice for 5 min and kept in methanol bath containing 0.5 per cent hydrogen peroxide at room temperature for 30 min for removing the endogenous peroxidase.

Then the slides were washed with Tris buffer twice and treated with HRPO labelled anti rabbit globulin (1:500 dilution, Sigma) for 45 min at 37°C.

The excess conjugate was removed by washing in tris buffer. Freshly prepared substrate solution in Tris-Hcl buffer in the presence of 0.01 per cent hydrogen peroxide was added and kept for 10 min at room temperature.

Finally the slides were examined under light microscope and the peroxidase activity was obtained as dark brown precipitate.

3.2.9 Indirect enzyme linked immunosorbent assay (ELISA)

Indirect ELISA was performed as per the procedure of Bernard *et al.* (1982) with slight modifications.

3.2.9.1 Antigen

Freeze dried canine distemper vaccine diluted with phosphate buffered saline (pH 7.4) was used as antigen.

3.2.9.2 Standardization of ELISA

The optimal concentration of coating antigen, sera dilution and peroxidase conjugated rabbit antidog globulin were standardized by the checker board titration and it was found that 1:400 dilution of antigen, 1:50 dilution of sera and 1:15000 dilution of peroxidase conjugated rabbit antidog globulin (Sigma) gave ideal result. The HRPO conjugate prepared in our laboratory did not give good results even at 1:10 dilution. The reason might be the addition of horse radish peroxidase enzyme in the conjugation procedure.

3.2.9.3 Test procedure

Microtitre plates of ninety six flat bottomed wells were coated with 1:400 diluted canine distemper antigen in carbonate-bicarbonate buffer (pH 9.6). 100 µl of antigen was placed in each well and plates were kept at 4°C overnight. At the end of coating, the plates were washed in PBST thrice and plates were stored at 4°C until used.

3.2.9.4 Application of bovine serum albumin

The stored plates were washed once with PBST and 100 µl of two per cent BSA was added to each well to block unreacted sites in the wells and incubated for one hour at 37°C. After incubation, plates were washed 3 times with PBST and dried by tapping gently against the filter paper.

3.2.9.5 Application of test sera

All the test sera were diluted to 1:50 using PBST. The samples were charged in six wells serially diluted from 1:50 to 1:1600. Each well was loaded with 100 µl. Positive and negative sera were also included in each run of the study. Serum was not added in substrate and peroxidase control. The plates were incubated for one hour at 37°C.

3.2.9.6 Addition of conjugate

At the end of incubation, the plates were washed in PBST thrice. Peroxidase conjugated antidog globulin (1:15000) was added 100 µl to each well. Peroxidase conjugate was not added in substrate control. The plates were incubated for one hour.

3.2.9.7 Addition of substrate

After one hour of incubation, the plates were washed thrice with PBST. All the wells were loaded with 100 µl of OPD substrate solution and incubated at room temperature for 20 minutes for the development of color reaction and the plates were read in Multiskan reader at 450 nm. Peroxidase control well was blanked to read the samples.

3.2.9.8 Interpretation of results

The mean optical density (OD) value which was twice and above the negative OD value was taken as positive. The sera of healthy dogs which were not vaccinated and were not infected were taken as negative control.

The optical density which was nearest to the sum of mean of negative control and three times the standard error was taken as the titre of the particular sample.

Results

Chapter IV

RESULTS

The canine distemper (CD) viral antigen in the lacrimal and nasal discharges of 113 clinically suspected dogs were studied using agar gel immunodiffusion (AGID) and counter immunoelectrophoresis (CIEP) and the results are presented in Table 1. The corneal impression smears were examined by using Giemsa and indirect immunoperoxidase for the detection of CD inclusion bodies from the above suspected dogs and the results are presented in Table 2.

The sera collected from 113 clinically suspected dogs and 190 apparently healthy dogs including vaccinated ones were examined for the presence of CDV antibodies using AGID and CIEP. The results are presented in Table 3. The sera were also screened for assessing the titre of antibodies to CDV by using indirect ELISA and the results are presented in Table 4.

4.1 Antigen detection

4.1.1 Agar gel immunodiffusion (AGID) test

Out of the 113 cases studied, lacrimal discharges from 13 cases (11.5 per cent) and nasal discharges from nine cases (7.96 per cent) of the clinically suspected dogs were positive for CDV antigen (Table 1, Fig.1, Plate 1) by AGID.

A two months old male Alsatian dog (O.P.No.17828) vaccinated against CD 10 days back and one two-year old male Alsatian dog (O.P. No.11218) vaccinated against CD 6 months back showed inappetance, pyrexia, purulent ocular and nasal discharge for about four days had CDV antigen in the lacrimal discharge.

A one and half year old male Doberman and a two months old female Doberman each having the CDV antigen in the nasal discharge showed pyrexia, inappetance, purulent eye and nasal discharges for about five days. The animals also showed diarrhoea and pustules on the abdomen.

4.1.2 Counter immunoelectrophoresis (CIEP)

Lacrimal discharges from nineteen cases (16.81 per cent) and nasal discharge from 9 cases (7.96 per cent) out of the 113 clinically suspected dogs were positive for CIEP (Table 1, Fig.1, Plate 2).

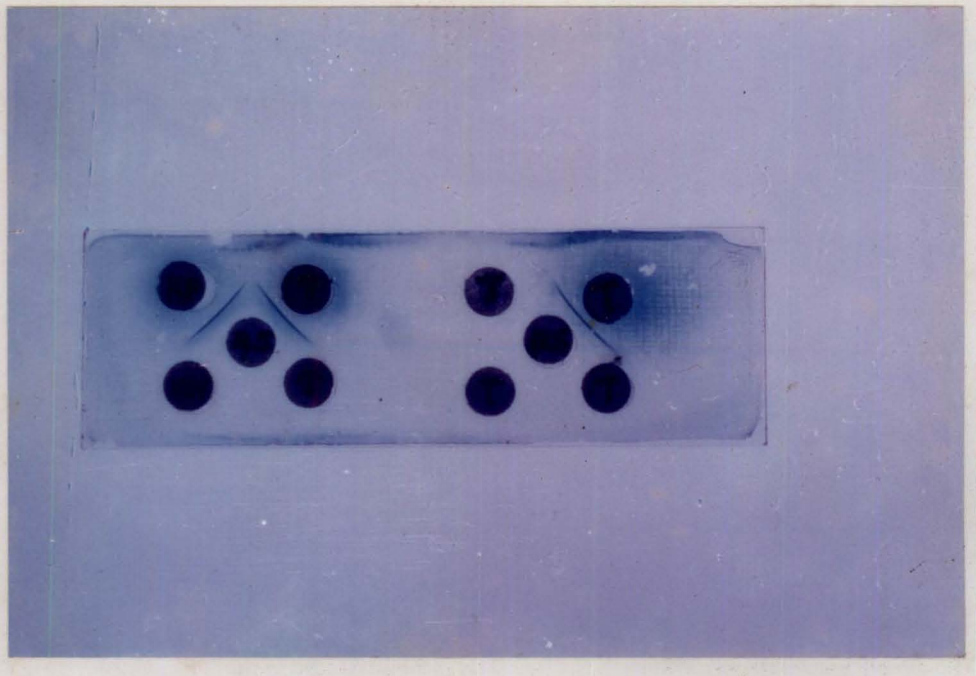
On analysing the clinical cases which were positive by CIEP, a female one year old crossbred female dog (O.P.No.12710) showed inappetance, vomition, diarrhoea and purulent ocular discharge for about two days and a one year old male Alsatian dog (O.P.No.18241) showed inappetance, congested mucous membrane, pyrexia, abdominal pustules, purulent ocular and nasal discharge.

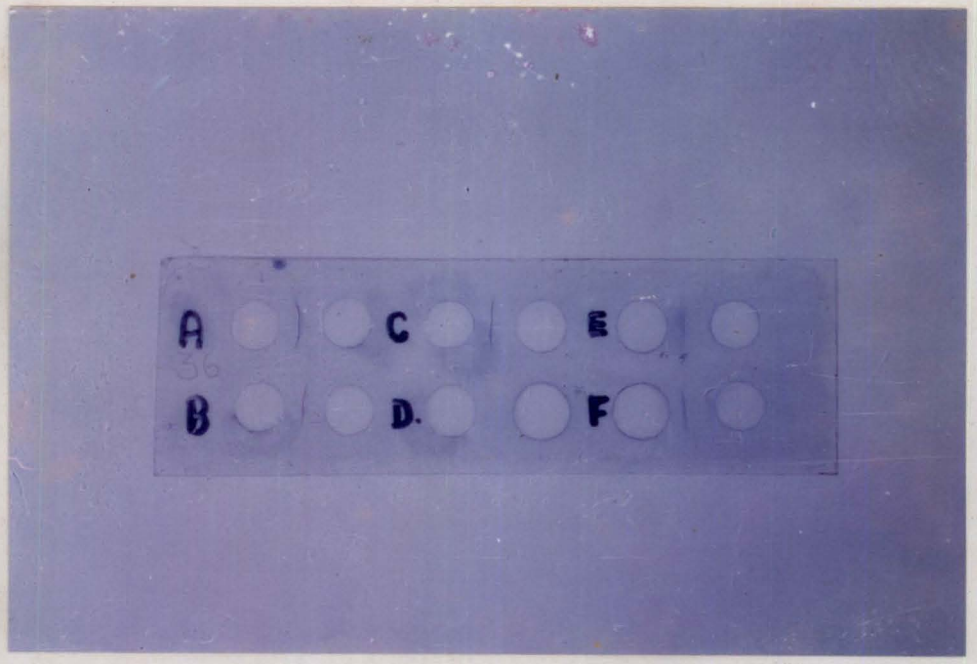
Plate 1. Agar gel immunodiffusion test - precipitation pattern of canine distemper viral antigen with sera samples

A : Concentrated antigen
KP : Known positive antigen
KN : Known negative serum
T : Test serum samples

Plate 2. Counter immunoelectrophoresis - precipitation pattern of canine distemper viral antigen with sera samples

A to F anode wells	:	Concentrated antigen
A,B,E and F cathode wells	:	Test sera samples
C cathode well	:	Positive control serum
D cathode well	:	Negative control serum




A
C
E
B
D
F

4.1.3 Giemsa staining

Out of 113 canine distemper suspected dogs, 4 (3.53 per cent) were having the CDV inclusion bodies in the corneal impression smears (Table 2, Fig.1, Plate 3).

Of these positive cases, a three year old male Alsatian crossbred dog (O.P.No.17964) and a three year old female Dachshund (O.P.No.14876) showed symptoms suggestive of canine distemper and O.P.No.11218 showing inappetance, congested mucous membrane, pyrexia and mucopurulent discharge from eyes was vaccinated 6 months back against canine distemper.

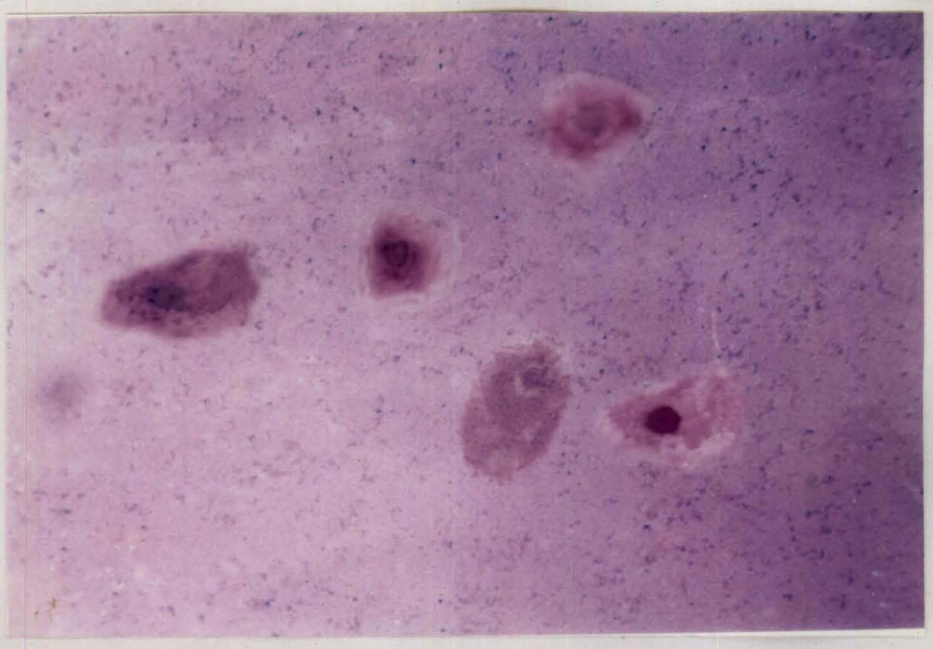
4.1.4 Indirect immunoperoxidase test

Out of 113 canine distemper suspected dogs, 71 (62.83 per cent) had canine distemper viral antigen viral antigen in the impression smears of cornea (Table 2, Fig.1, Plate 4).

Among the positive cases, a two year old female Alsatian (O.P.No.5803) and one eight month old male Alsatian dog (O.P.No.7976) which was vaccinated 15 days back against canine distemper showed inappetance, pyrexia and purulent ocular discharge for about two days but the appetite was normal in C.5803.

Plate 3. Giemsa staining of corneal impression smear showing esinophilic intracytoplasmic inclusion body x400

Plate 4. Indirect immunoperoxidase staining of corneal impression smear showing brown deposits in the nucleus and cytoplasm x1200



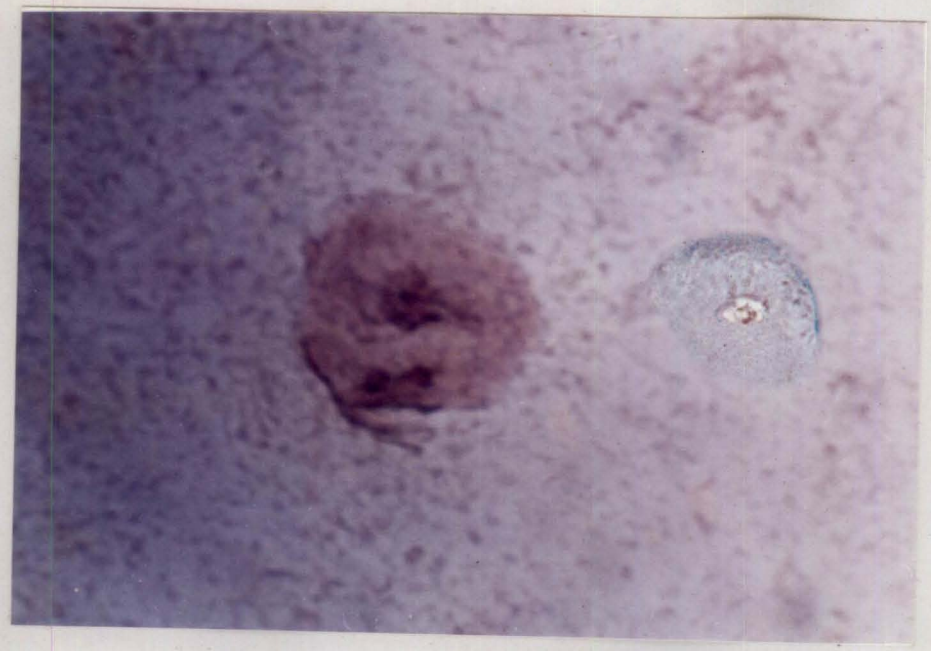


Table 1. Detection of canine distemper viral antigen by AGID and CIEP

Type of materials collected	Numbers of animals tested	AGID		CIEP	
		Positive	Percentage	Positive	Percentage
Lacrimal discharge	113	13	11.50 NS	19	16.81 NS
Nasal discharge	113	9	7.96 NS	9	7.96 NS

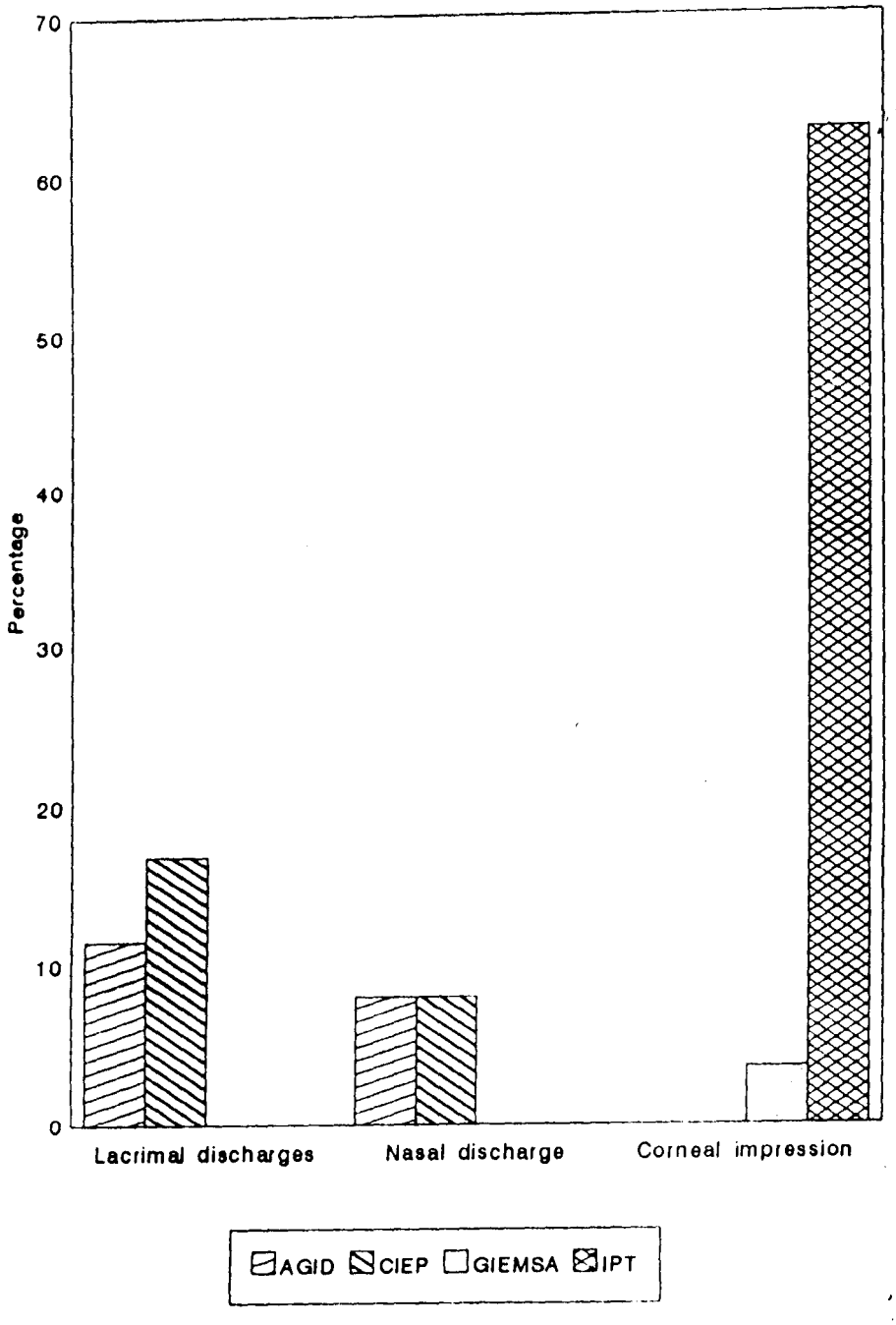
NS - Non-significant

Table 2. Detection of canine distemper viral antigen by Giemsa statining and indirect immunoperoxidase test

Type of materials collected	Numbers of animals tested	Giemsa staining		Immunoperoxidase test	
		Positive	Percentage	Positive	Percentage
Corneal impression smears	113	4	3.53	71	62.83*

* Highly significant ($P \leq 0.01$)

Fig.1 DETECTION OF CANINE DISTEMPER VIRAL ANTIGEN BY DIFFERENT SEROLOGICAL TESTS



4.2 Antibody detection

4.2.1 Agar gel immunodiffusion test

Among 303 animals, including healthy vaccinated dogs, nine (7.96 per cent) were positive for canine distemper viral antibodies (Table 3, Fig.2).

A two year old female Pomeranian dog (O.P.No.7955) showing shivering for about two months and a three year old male non-descript dog showing congested mucous membrane, temporal twitching, severe convulsions, staggering gait, champing of jaw gave positive results with AGID.

4.2.2 Counter immuno electrophoresis

Sera from nine dogs (7.96 per cent) out of the 303 tested were positive for canine distemper antibodies.

One, two year old male non-descript dog (O.P.No.13635) showing congested mucous membrane, muscle twitching, champing, of jaw and fits for about ten days and a three year old male Alsatian dog showing inappetance, congested mucous membrane and shivering gave positive results with CIEP.

4.2.3 Indirect ELISA

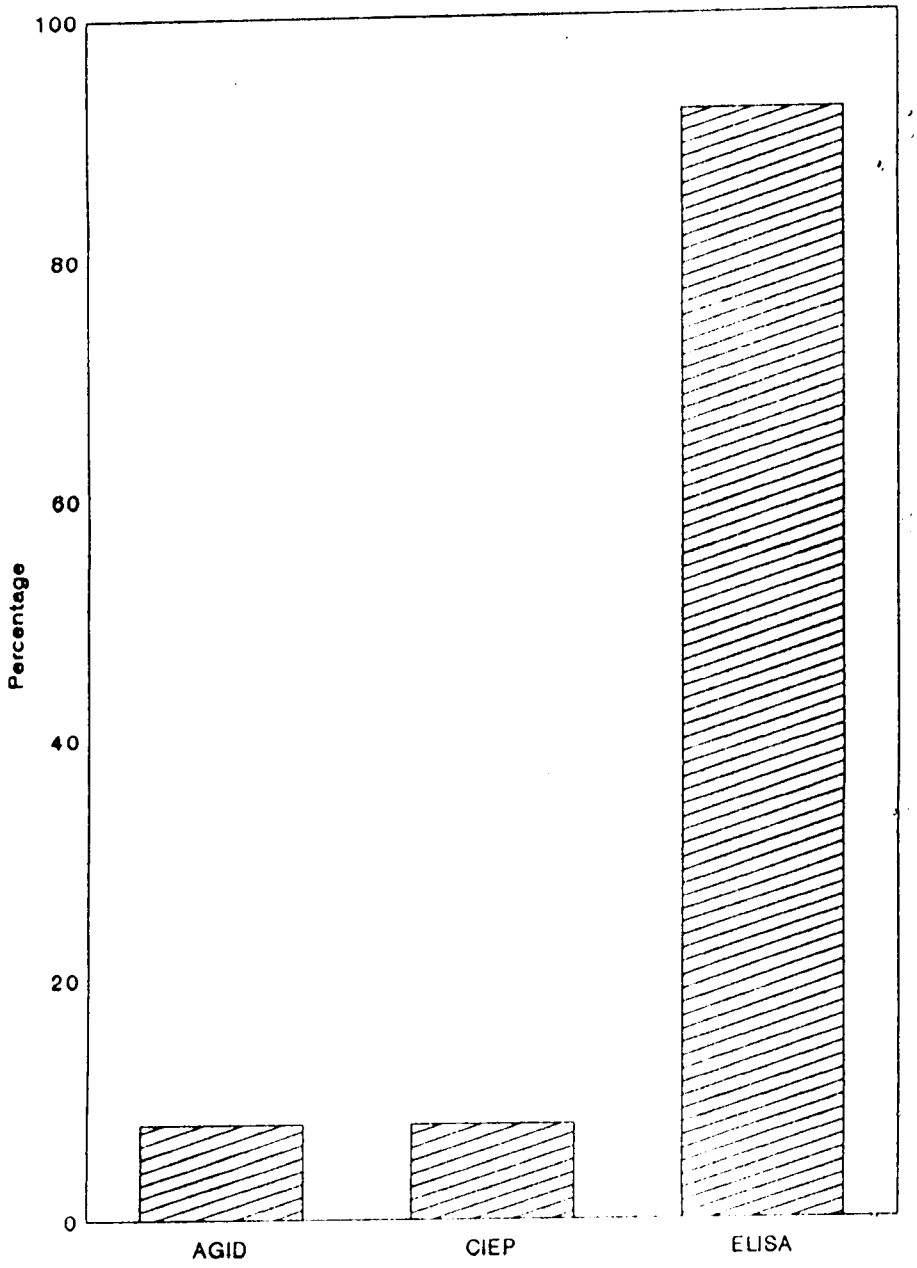
The sera collected from 113 canine distemper suspected and 190 apparently healthy dogs including vaccinated ones were

Table 3. Detection of antibody to canine distemper viral antigen by AGID and CIEP

Type of animals	Tested	AGID		CIEP	
		Positive	Percentage	Positive	Percentage
CD suspected dogs	113	9	7.96 NS	9	7.96 NS
Vaccinated dogs	17	-	-	-	-
Healthy dogs	173	-	-	-	-

NS - Non-significant

Fig.2 DETECTION OF CANINE DISTEMPER VIRAL ANTIBODY BY DIFFERENT SEROLOGICAL TESTS



screened for antibody to CDV and the result is presented in Table 4, Fig.3 and Plate 5.

4.2.3.1 Canine distemper suspected dogs

Out of the 113 clinically suspected cases, 104 (92.03 per cent) dogs showing the titre of ≥1:100 were positive and nine (7.96 per cent) dogs showing the titre of 1:50 were negative for antibody to CDV (Table 4).

Among the 104 seropositive dogs, 22 (21.15 per cent) 18 (17.3 per cent), 11 (10.5 per cent), 17 (16.34 per cent) and 36 (34.6 per cent) dogs were showing the antibody titre of 1:100, 1:200, 1:400, 1:800 and 1:1600, respectively. Of these positive cases, one 12 year old female labrdador crossbred dog (O.P.No.19973) and a two months old male Alsatian (O.P.No.17828) which were vaccinated 2 years and 6 months back respectively, showed inappetance, pyrexia, purulent discharges from eyes and nostrils, abdominal pustules, diarrhoea, vomition and cough. Three animals, a three year old male Alsatian, a male non-descript two year old and another Alsatian male one and half year old with high antibody titres ranging from 1:800 and 1:1600 showed inappetance, pyrexia, pneunomia, purulent ocular and nasal discharge, abdominal pustules and nervous signs of temporal twitching, champing of jaw, severe convulsion and staggering gait for about a week.

4.2.3.2 Vaccinated dogs

Of the sera samples collected from 17 canine distemper vaccinated dogs, 15 (88.23 per cent) cases were positive for CD antibodies with the titre ranging from 1:100 to 1:400 and two (11:76 per cent) were negative showing the titre of 1:50. Among the 15 seropositive dogs, 3 (20 per cent), 5 (33.33 per cent) and 7 (46.66 per cent) cases had the antibody titres of 1:100, 1:200 and 1:400 respectively.

4.2.3.3 Healthy dogs

The sera samples collected from 173 healthy dogs which were not vaccinated against CD were screened for assessing the antibody titre.

Out of the 173 healthy dogs, 83 (47.97 per cent) cases were positive for antibody to CDV. Among this above seropositive dogs, 36 (43.37 per cent), 33 (39.75 per cent) and 14 (16.86 per cent) cases were showing the antibody titre of 1:100, 1:200 and 1:400, respectively.

4.3 Incidence

4.3.1 Age

Among the 202 seropositive dogs given in Table 5, 81 cases (40.09 per cent) were in the age group 0-1 year, followed by the 1-3 year group with 66 cases (32.67 per cent), the 3-5 year

Plate 5. Indirect ELISA

A row 1		**:**	**Substrate control**
2,3		**:**	**HRPO control**
4 to 6		**:**	**Negative control**
7 to 12		**:**	**Positive serum control (serially diluted from 1:50 to 1:1600)**
B to H row	**1 to 6**	**:**	**Test serum samples (serially diluted from 1:50 to 1:1600)**
	7 to 12		

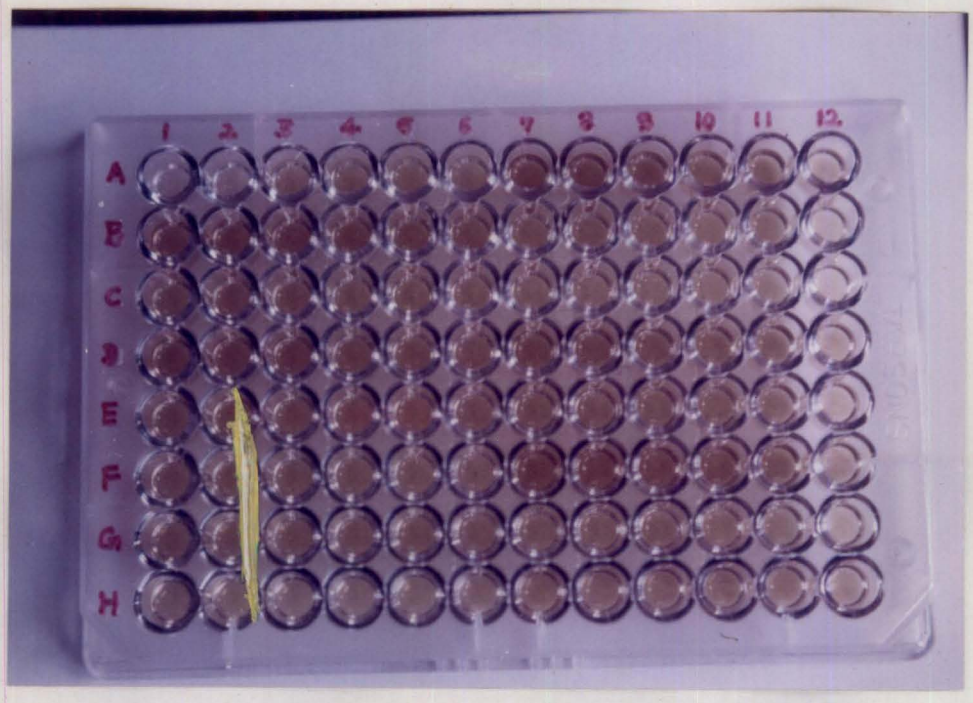
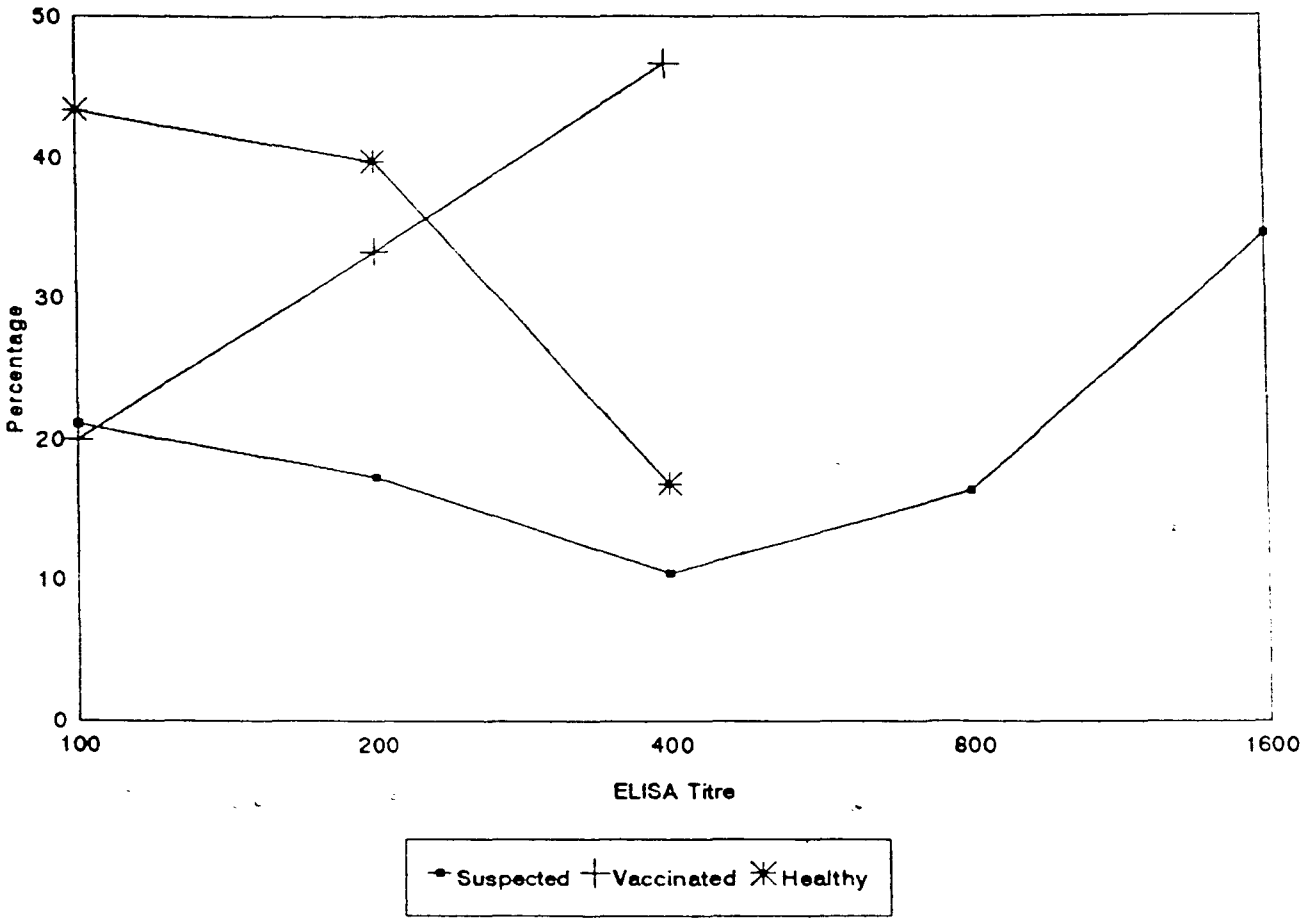

1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

Table 4. Indirect ELISA-antibody tirre to canine distemper virus among seropositive dogs

Type of animal	1:50	1:100	1:200	1:400	1:800	1:1600	Total	Total positive	Total negative	Percentage positive	Percentage negative
CD suspected dogs	9	22 (21.15%)	18 (17.3%)	11 (10.57%)	17 (16.34%)	36 (34.61)	113	104	9	92.03	7.96
Vaccinated dogs	2	3 (20%)	5 (33.33%)	7 (46.66%)	-	-	17	15	2	88.23	11.76
Healthy dogs	90	36 (43.37%)	33 (39.75%)	14 (16.86%)	-	-	173	47.97	90	48	52
Total	101	61	56	32	17	36	303	202	101	67	33
Per cent	33.33	20.13	18.48	10.56	5.61	11.88		66.66			
			33.33%			66.66%					
			Negative			Positive					

Fig. 3 ELISA ANTIBODY TITRE TO CANINE DISTEMPER VIRUS AMONG SEROPOSITIVE DOGS



group with 38 cases (18.81 per cent), the 5-10 year group with 14 cases (6.93 per cent) and 10-15 year group with 3 cases (1.48 per cent).

4.3.2 Sex

Number of seropositive cases were more among males with 125 (61.88 per cent) positive ones among 202 compared to that among females with 77 (38.11 per cent) positive as shown in Table 6 and Figure 5.

4.3.3 Breed

More sero positive dogs noted among 202 positive cases (Table 7) were Alsatian 61 (30.19 per cent) followed by Pomeranian 35 (17.32 per cent), non descript dogs 32 (15.84 per cent), Dachshund 17 (8.42 per cent), Doberman 13 (6.43 per cent), Labrador 7 (3.46 per cent), Great Dane 5 (2.47 per cent), Irish Setter 3 (1.48 per cent), Lhasa Apso 3 (1.48 per cent), Cocker Spaniel 2 (0.99 per cent), Dalmatian 2 (0.99 per cent), Weimaraner 2 (0.99 per cent), Afghanhound 1 (0.49 per cent), Boxer 1 (0.49 per cent), Rottweiler 1 (0.49 per cent) and Crossbreds of 17 (8.42 per cent) and the result is presented in Table 7 and Figure 6.

4.3.4 Seasonal variation

Among the 202 seropositive dogs given in Table 8 and Figure 7, the number of cases recorded during May were 26 dogs

Table 5. Age distribution of canine distemper sero positive dogs

Age years	Number seropositive	Percentage
0-1	81	40.09**
1-3	66	32.67**
3-5	38	18.81
5-10	14	6.93
10-15	3	1.48
Total	202	

** Highly significant (P≤0.01)

Table 6. Sex wise distribution of canine distemper seropositive dogs

Sex	Number seropositive	Percentage
Male	125	61.88*
Female	77	38.11
Total	202	

* Significant (P≤0.05)

Fig. 4 AGE DISTRIBUTION OF CANINE DISTEMPER SEROPOSITIVE DOGS

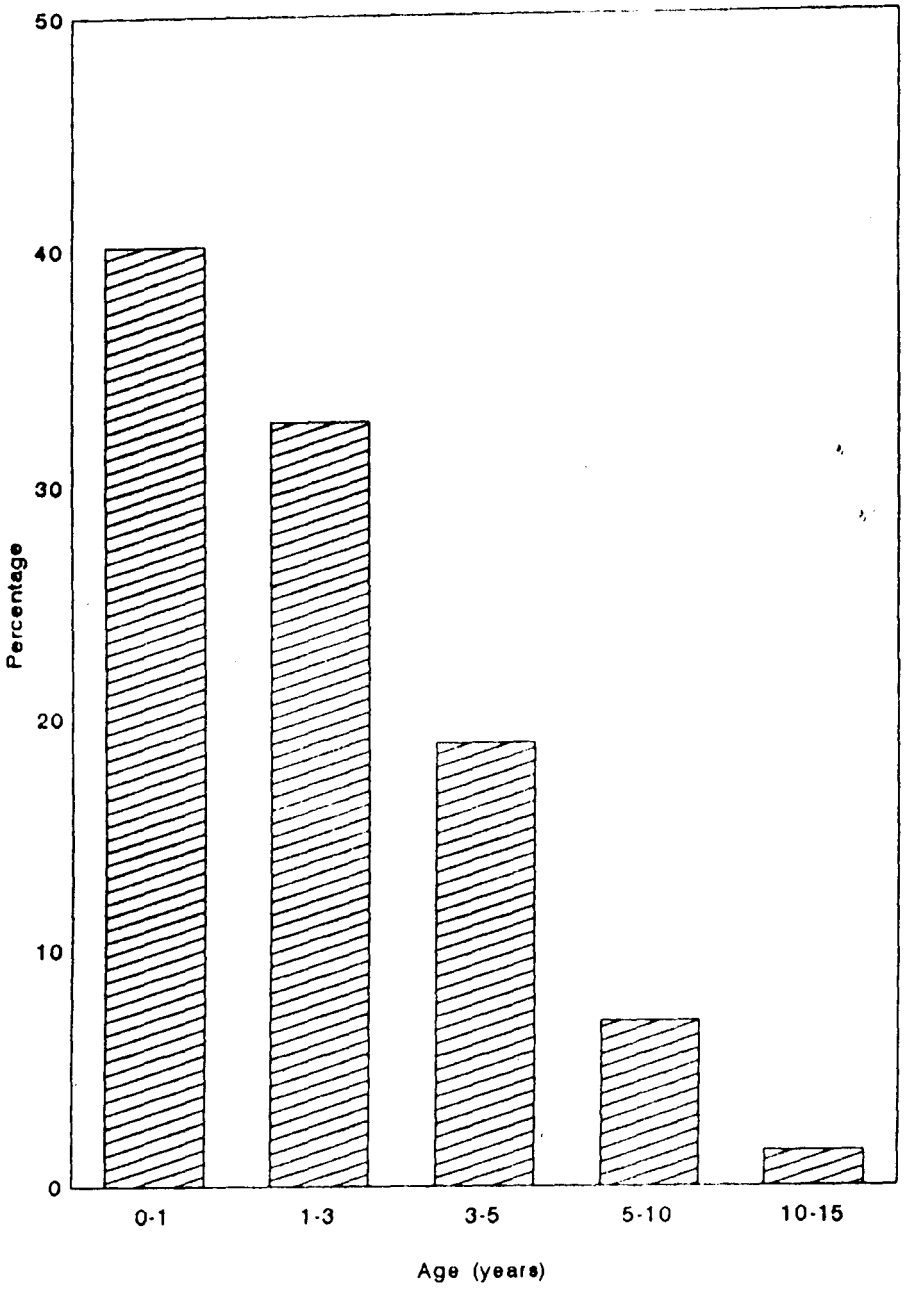


Fig. 5 SEX DISTRIBUTION OF CANINE DISTEMPER SEROPOSITIVE DOGS

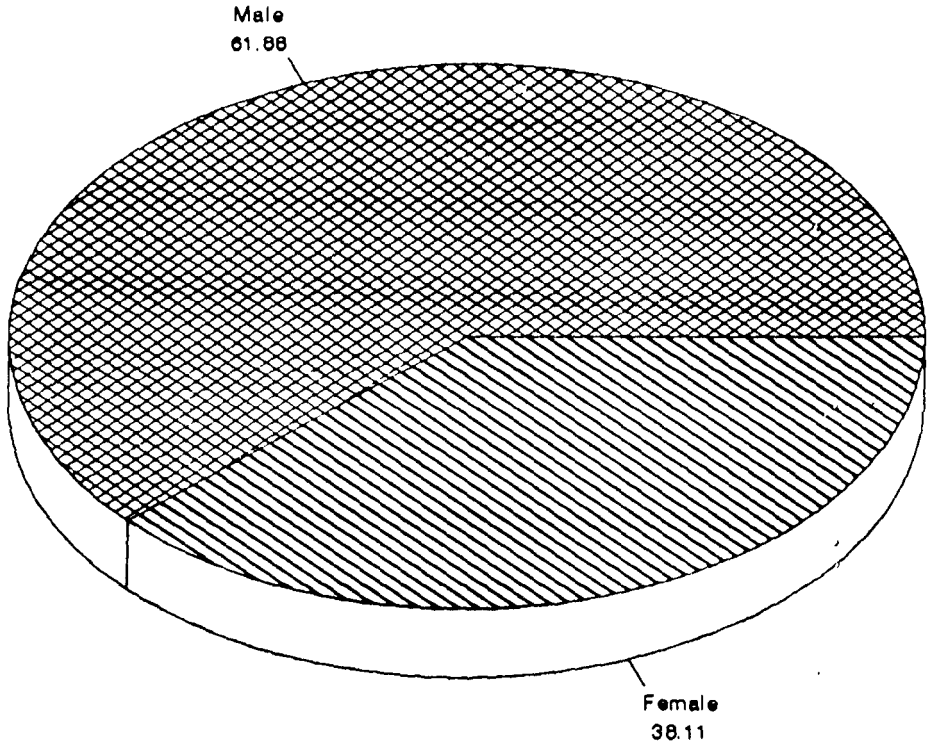
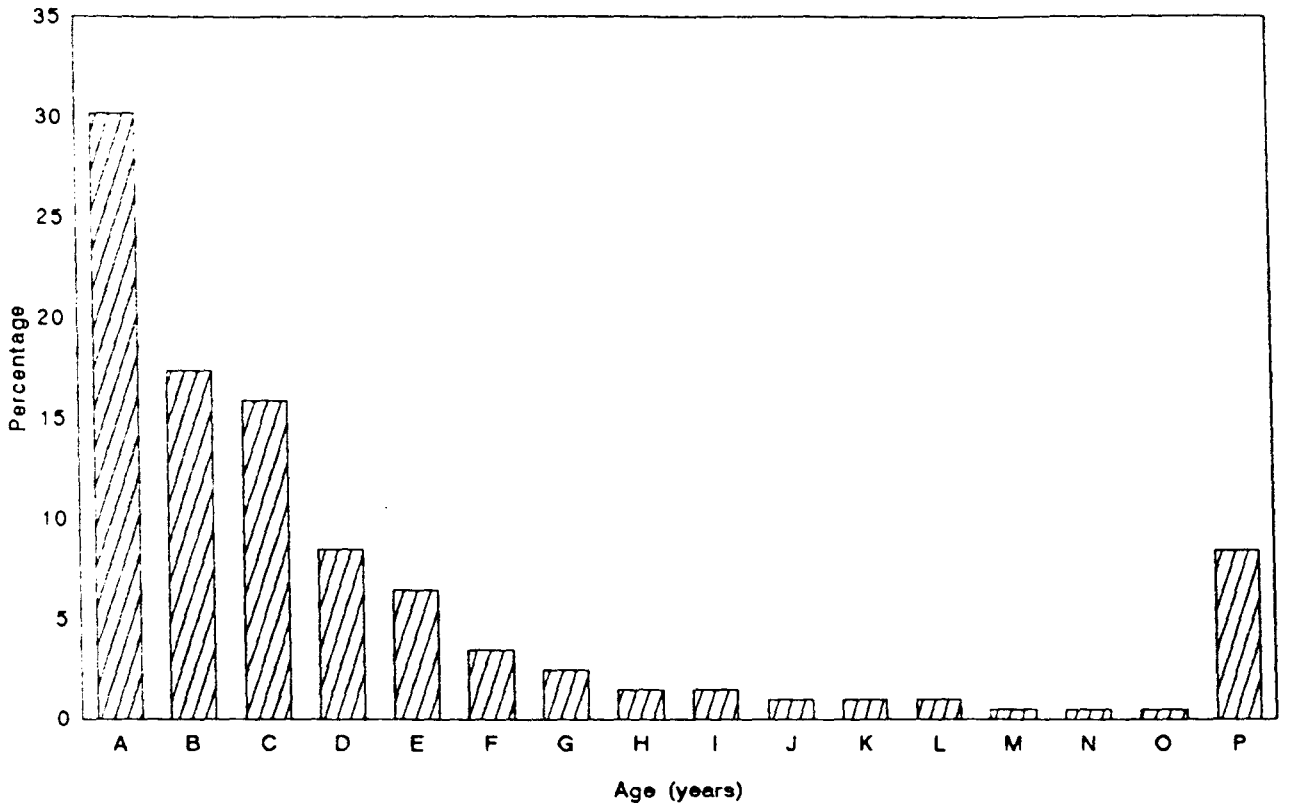


Table 7. Breed wise distribution of canine distemper seropositive dogs

Breed	Number seropositive	Percentage
Alsatian	61	30.19*
Pomeranian	35	17.32
Non descript	32	15.84
Dachshund	17	8.42
Doberman	13	6.43
Labrador	7	3.46
Great dane	5	2.47
Irish setter	3	1.48
Lhasa Apso	3	1.48
Cocker Spaniel	2	0.99
Dalmatian	2	0.99
Weimaraner	2	0.99
Afghan Hound	1	0.49
Boxer	1	0.49
Rottweiler	1	0.49
Cross breeds	17	8.42
Total	202	

* Significant ($P \leq 0.05$)

Fig. 6 BREED WISE DISTRIBUTION CANINE DISTEMPER SEROPOSITIVE DOGS



A-Alsatian;B-Pomeraninan;C-Non descript;D-Dachshund;E-Doberman
F-Labrador;G-Great Dane;H-Irish setter;I-Lhasa Apso;J-Cocker Spaniel
K-Dalmatian;L-Weimaraner;M-Afghan Hound;N-Boxer;O-Rottweiler;P-Cross breeds.

(12.87 per cent), June - 25 dogs (12.37 per cent), July - 23 dogs (11.38 per cent), January - 22 dogs (10.89 per cent), February - 20 dogs (9.9 per cent), November - 18 dogs (8.91 per cent), March - 16 dogs (7.92 per cent), August - 14 dogs (6.93 per cent), December - 14 cases (6.93 per cent), April 12 dogs (5.94 per cent) and October - 10 dogs (4.95 per cent).

4.4 Clinical signs

Out of the 104 canine distemper positive cases given in Table 9, 85 (81.73 per cent), 101 (97.11 per cent), 64 (61.53 per cent), 14 (13.46 per cent), 8 (7.69 per cent), 39 (37.5 per cent), 14 (13.46 per cent), 45 (43.26 per cent), 14 (13.46 per cent), 1 (0.96 per cent) showed pyrexia, inappetance, purulent lacrimal discharge, purulent nasal discharge, respiratory distress, vomition, diarrhoea, nervous signs, abdominal pustules, hyperkeratosis of foot pad and nose, respectively.

Table 8. Month wise distribution of canine distemper seropositive dogs

Month	Year		Percentage	
	1997	1998	1997	1998
January	-	22	-	10.89 NS
February	-	20	-	9.90 NS
March	-	16	-	7.92 NS
April	-	12	-	5.94 NS
May	-	26	-	12.87 NS
June	-	25	-	12.37 NS
July	23	-	11.38 NS	-
August	14	-	6.93 NS	-
September	2	-	0.99 NS	-
October	10	-	4.95 NS	-
November	18	-	8.91 NS	-
December	14	-	6.93 NS	-
Total	85	117		

NS : Non-significant

Fig. 7 MONTH WISE DISTRIBUTION OF CANINE DISTEMPER SEROPOSITIVE DOGS

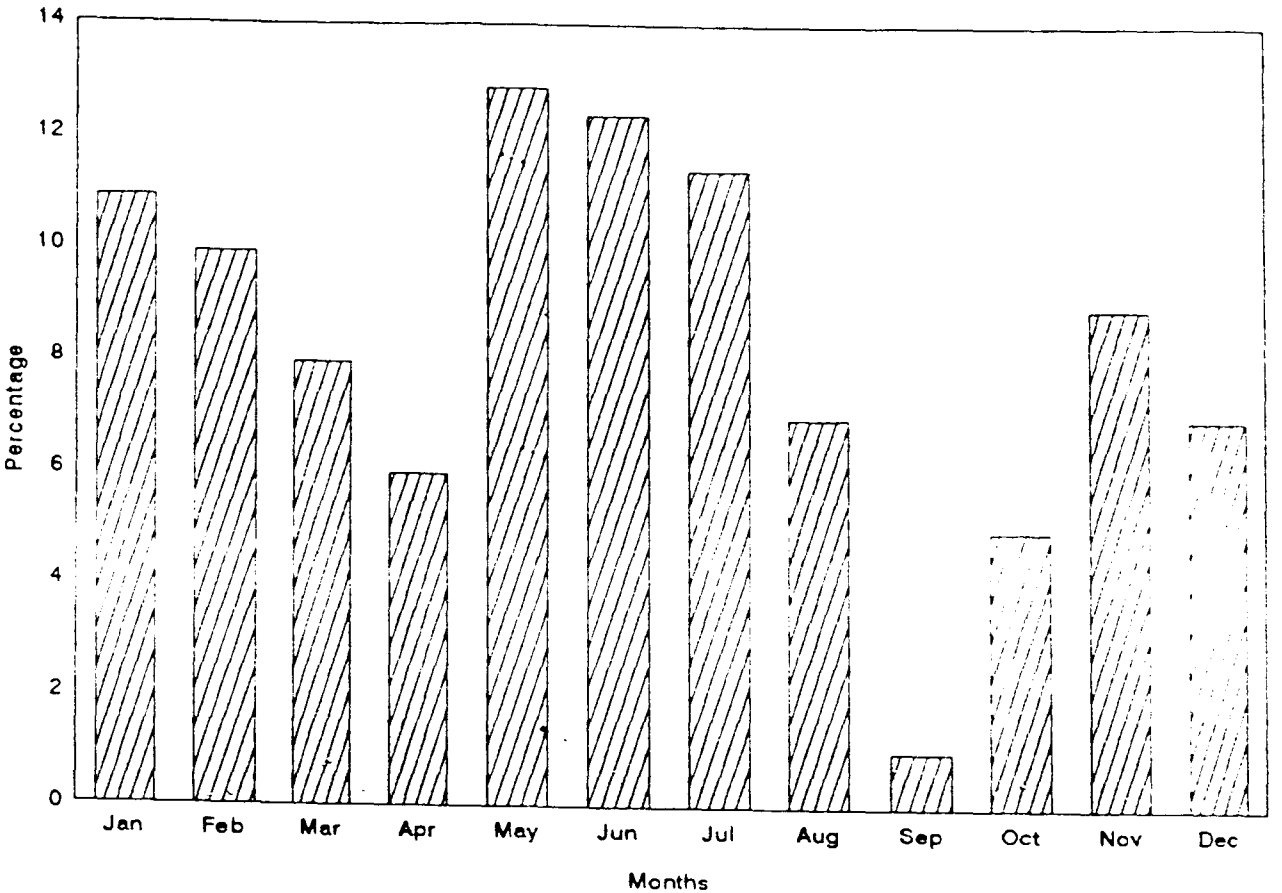


Table 9. Relationship of clinical symptom to canine distemper

Clinical symptom	Positive to CD	Per cent
Pyrexia	85	81.73
Inappetance	101	97.11
Purulent eye discharge	64	61.53
Purulent nasal discharge	14	13.46
Respiratory distress	8	7.69
Vomition	39	37.50
Diarrhoea	14	13.46
Nervous symptoms (temporal twitching, champing of jaw, convulsion, shivering, staggering gait)	45	43.26
Abdominal pustules	14	13.46
Hyperkeratosis of foot pad and nose	1	0.96
Clinically healthy for vaccination	15	14.42

Discussion

Chapter V

DISCUSSION

In the present study, the prevalence of canine distemper (CD) in Thrissur district was assessed. The lacrimal discharge and nasal discharge of one hundred and thirteen CD suspected dogs were subjected to Agar gel immunodiffusion (AGID) test and Counter immuno electrophoresis (CIEP) test. The corneal impression smears of the above suspected dogs were screened for the detection of canine distemper inclusion bodies and CD viral antigen using Giemsa staining and indirect immunoperoxidase test (IPT), respectively. A total of three hundred and three dogs were tested for CD viral antibody by AGID and CIEP. The CD viral antibody was also assessed using indirect Enzyme Linked Immunosorbent Assay (ELISA).

5.1 Antigen detection

5.1.1 Agar gel immunodiffusion (AGID) test

Agar gel immunodiffusion test detected canine distemper viral antigen in the lacrimal discharge of 11.5 per cent cases and in the nasal discharge of 7.96 per cent of canine distemper suspected dogs (Table 1, Fig.1, Plate 1).

The positive animals were showing pyrexia, anorexia, purulent ocular discharge and nasal discharge, vomition and diarrhoea.

Ramadass and Balu (1982) had detected canine distemper viral antigen in 57.1 per cent canine distemper suspected using lacrimal discharge by AGID and the positive dogs were showing only gastroenteritis. Ganesan *et al.* (1984) observed CD virus antigens in the lacrimal discharges of 74 per cent dogs which were showing mucopurulent lacrimation, enteritis, pneumonia and nervous symptoms. However, the results obtained in the present study were not agreeing with their findings. The low percentage of antigen detected by AGID could be either due to the low sensitivity of the test or due to the stage of the disease when the collection of lacrimal and nasal discharge was done. Cornwell *et al.* (1965) reported that the canine distemper viral excretion was more or less between 10-13 days after exposure to infection.

5.1.2 Counter immunoelectrophoresis (CIEP)

Counter immunoelectrophoresis detected canine viral antigen in the lacrimal discharge of 16.81 per cent cases and in nasal discharge of 7.96 per cent of CD suspected dogs and the results are presented (Table 1, Fig.1, Plate 2).

On analysing the positive cases by CIEP, the symptoms exhibited were inappetance, pyrexia, anorexia, purulent ocular and nasal discharge, vomition, diarrhoea and abdominal pustules. Ramadass *et al.* (1983) had detected canine distemper viral antigens in 78.57 per cent and 75 per cent of CD

suspected dogs using lacrimal discharge by CIEP and AGID, respectively. Similar findings were not coincided with the results of present study probably due to the stage of the disease and the time of collection of lacrimal and nasal discharge.

Eventhough, the difference was non-significant between CIEP and AGID in the present study, a higher percentage of CD viral antigens were detected by CIEP than AGID, which was also stated by Ramadass *et al.* (1983).

5.1.3 Giemsa staining

Demonstration of canine distemper viral inclusions is one of the diagnostic tests for canine distemper. In the present study, the CDV inclusion bodies could be detected in 3.53 per cent cases (Table 2, Fig.1, Plate 3) of the corneal impression smear of dogs clinically suffering from distemper and the positive animals showed inappetance, pyrexia, congested mucous membrane, vomition and diarrhoea.

Many workers (Schalm *et al.*, 1975; Gossett *et al.*, 1982; Jones and Hunt, 1982 and Smith *et al.*, 1983) demonstrated canine distemper inclusion bodies in the blood cells whereas the present study conforms with the observation of Ocampo (1972) who stated that canine distemper viral inclusion bodies could not be detected in the cells of corneal impression smear

in all canine distemper suspected dogs. Cornwell *et al.* (1965) detected more number of inclusion bodies 11-13 after exposure to infection in dogs and also stated that examination of conjunctival smears for the presence of inclusion bodies was of limited diagnostic value due to the small number of cells detached and comparatively late appearance of inclusion bodies. Schalm *et al.* (1975) and Cello *et al.* (1959) had reported that the inclusions were difficult to find and stained poorly with routine stains. Chandler *et al.* (1991) stated that the examination of conjunctival smear for the detection of CD inclusion bodies by conventional staining was insensitive and difficult to interpret due to the insufficiency of cells and the difficulty of distinguishing viral from other type of inclusions in the small number of rather degenerate cells.

Though the finding of characteristic inclusion bodies in CD suspected dogs is a confirmatory test, the poor result obtained in the present study indicate that this is not a reliable test.

5.1.4 Indirect Immunoperoxidase test (IPT)

Indirect immunoperoxidase test detected canine distemper viral antigens in 62.83 per cent of CD suspected dogs using corneal impression smear (Table 2, Fig.1, Plate 4) and the positive animals showed inappetance, pyrexia and purulent ocular discharge. The present finding agrees with the

observation of Ductalle *et al.* (1980) and Machida *et al.* (1993) who demonstrated canine distemper viral antigens in 78.57 per cent and 88.87 per cent dogs respectively using the tissue sections of CD suspected dogs, by IPT.

Based on the result obtained in the present study, the indirect immunoperoxidase test is considered to be a reliable test for the diagnosis of canine distemper.

5.2 Antibody detection

5.2.1 Agar gel immunodiffusion (AGID) test

In the present study, canine distemper viral antibodies were detected in 7.96 per cent cases of CD suspected dogs by AGID. The sera collected from 190 apparently healthy dogs were negative for CDV antibody by this method (Table 3, Fig.2).

The positive animals showed the signs of inappetance, pyrexia, cough, vomition and nervous signs of champing of jaw, temporal twitching, severe convulsions shivering and staggering gait. The animals which showed nervous signs suggestive of distemper and having the required antibody titre by indirect ELISA in the present study, were positive by AGID also.

The presence of antibody in clinically suggestive canine distemper cases in the present study confirms with the observation of Motohashi *et al.* (1961) who stated that the

canine distemper virus may be active even in the presence of antibody because at any given time a proportion of virus is intracellular and they found high levels of antibody in severe cases of distemper also.

5.2.2 Counter immunoelectrophoresis (CIEP)

Counter immunoelectrophoresis detected canine distemper viral antibodies in 7.96 per cent of dogs suffering from distemper (Table 3, Fig.2). The sera samples from apparently healthy dogs were negative for CDV antibody by CIEP.

The positive animals showed inappetance, pyrexia, coughing, vomition, diarrhoea and nervous signs, suggestive of canine distemper and had high titre of CD viral antibodies by indirect ELISA test. Oldstone and Fujinami (1982) proposed a hypothetical mechanism in which the presence of specific antibody during infection induced antigenic modulation and redistributed the antigens so as to reduce their expression on the infected cell surface resulting in viral persistence.

5.2.3 Indirect ELISA

Out of the 303 dogs screened for canine distemper, 66.66 per cent of the dogs including CD suspected and healthy vaccinated ones had the protective level of antibody (>1:100) against canine distemper virus (Table 4, Fig.3, Plate 5).

5.2.3.1 Canine distemper suspected dogs

The clinically positive animals which had the antibody titre of more than 1:100 in ELISA showed pyrexia, anorexia, purulent ocular and nasal discharge, abdominal pustules, vomition, diarrhoea and nervous signs characteristic of distemper.

The antibody titres of 1:100, 1:200 and 1:400 in 21.15 per cent, 17.3 per cent and 10.5 per cent of CD suspected dogs were due to the different stages of infection at the time of blood collection. The animals in the early stage of infection showing the symptoms of CD for about 2 days had the antibody titre of 1:100 and the animals in mid stage of infection showing the symptoms of CD for about 3-5 days had the antibody titre of 1:200 and 1:400. The high antibody titre of 1:800 and 1:1600 present in 16.34 per cent and 34.6 per cent cases, respectively showing inappetance, pyrexia, abdominal pustules, purulent ocular and nasal discharge, nervous signs of temporal twitching, shivering, convulsions for about 7-10 days were due to the late stage of infection.

Appel and Gillespie (1972) had stressed that a rapid and high titred viral antibody response to CDV was crucial to recovery from viral infection. Krakowka *et al.* (1985) suggested that the viral antibody was not invariably protective

and it facilitated the penetration of virus into CNS tissues in severe cases which resulted in persistence of the virus.

5.2.3.2 Vaccinated animals

Subjecting the sera collected from dogs vaccinated against distemper to indirect ELISA test 88.23 per cent dogs had protective level of antibody titre while 13.33 per cent had titre below 1:100. the reason for low level of titre could be due to immunity breakdown either due to vaccine factor or host factor. Povey . . . . (1986) discussed about canine distemper vaccine failure which occurred due to maternal and hereditary influences, exposure to intercurrent infections, drug administration or surgery concurrent with vaccination, physical environment, nutrition and exposure to virulent virus.

5.2.3.3 Healthy dogs

Of the one hundred and seventy three healthy dogs brought for general check-up, deworming and vaccination, 20.80 per cent, 19.07 per cent and 8.09 per cent had the antibody titres of 1:100, 1:200 and 1:400 respectively against distemper.

The probable reason for apparently healthy dogs which were having the protective level of antibody against canine distemper in the present study is that they might have been infected earlier subclinically with canine distemper virus and the animal became immune to the disease. Appel and Carmichael

(1979) had reported CD in 75 per cent of dogs suffering subclinically.

ELISA had proven to be more specific and sensitive in detecting antibodies against CDV (Noon *et al.*, 1980; Blixenkrone-Moller *et al.*, 1993 and John *et al.*, 1996). Similar findings were also observed in the present study.

Out of the 303 dogs screened from Thrissur district, the prevalence of canine distemper was 66.66 per cent by using ELISA. The animals which were positive by AGID and CIEP had high ELISA antibody titre of 1:1600 and the test provided an excellent method for measuring the CD viral antibody.

5.3 Incidence

5.3.1 Age

The dogs in the age group of 0-1 year and 1-3 year recorded in the present study were more affected by CDV compared to other age group dogs and a significant difference ($P<0.01$) in the number of seropositive dogs was noticed between the age groups. These above findings are in agreement with the reports of Hofman (1949); Appel and Carmichael (1979), Ramadass and Balu (1982), Ogunkoya *et al.* (1985); Pop *et al.* (1989) and Malik *et al.* (1995). The findings were shown in Table 5, Fig.4.

5.3.2 Sex

More number of male dogs were seropositive than females as shown in Table 6 and Fig.5 and a significant difference was obtained between the sexes ($P<0.05$). Similar findings were observed by Lounela *et al.* (1997). Morales *et al.* (1997) reported that both sexes were equally affected to CDV. Probably the high population of male dogs could be the reason for the present finding.

5.2.3.3 Breed

The breed wise incidence of canine distemper in the present study (Table 7, Fig.6) was found to be higher in the Alsatian compared to other breeds and the results indicated a significant difference in seroprevalence between the breeds ($P<0.05$). Similar findings have been described by Ganesan *et al.* (1985) and Saseendranath *et al.* (1997). Noon *et al.* (1980) stated that all breeds were equally susceptible to CD. Patronek *et al.* (1995) reported that the risk of disease for pure bred dogs was more than the mixed breed dogs. The high population of Alsatian dogs in Thrissur district might be one important factor in the present finding.

5.2.3.4 Season

On the analysis of the results, more number of seropositive dogs in the present study were recorded during

May, June and July followed by January and February. Otherwise, the incidence was evenly spread out throughout the year. The difference among months was not significant as shown in Table 8, Fig.7.

This finding is in accordance with Ganesan *et al.* (1985). Anonymous (1963) reported the incidence of canine distemper at the onset of inclement weather in the fall whereas Roscoe (1993) recorded canine distemper infection in summer. Some authors (Erno and Moller, 1961; Appel and Carmichael, 1979) found no seasonal variation in the occurrence of canine distemper among dog population. The present finding reveals that the attack rate is constant throughout the year but the clinical disease manifests more frequently in May, June and July.

Summary

Chapter VI

SUMMARY

The prevalence of canine distemper in three hundred and three dogs in Thrissur district was studied. The canine distemper (CD) viral antigen and CD viral antibody were detected for the diagnosis of the case. CD viral antigen were detected in nasal discharge, lacrimal discharge by using AGID and CIEP. CD viral inclusion bodies and CD viral antigen were also detected in impression smears of cornea by Giemsa staining and indirect immunoperoxidase test (IPT), respectively. Caine distemper viral antibody was detected by using AGID and CIEP and the antibody titre was assessed by indirect ELISA.

Agar gel immunodiffusion test detected CD viral antigen in the lacrimal discharge of 11.5 per cent and in the nasal discharge of 7.96 per cent dogs. The above suspected dogs were also tested for detection of CD viral antigen by CIEP and it was found that 16.8 per cent and 7.96 per cent dogs had the CD viral antigen in the lacrimal and nasal discharge, respectively. There was no significant difference between AGID and CIEP.

Canine distemper inclusion bodies were present in 3.53 per cent corneal impression smears of CD suspected dogs by Giemsa staining. The corneal impression smear was also subjected for detection of CD viral antigen by using indirect

immunoperoxidase test and 62.83 per cent of CD suspected dogs showed positive reaction. A significant difference ($P \leq 0.01$) was observed between Giemsa staining and indirect IPT.

The agar gel immunodiffusion test was also used to detect the CD viral antibody in sera. Out of 303 dogs screened, including 190 apparently healthy and vaccinated dogs, the CD viral antibody was present in 7.96 per cent suspected dogs whereas counter immunoelectrophoresis detected CD viral antibody in 7.96 per cent cases of CD suspected dogs. Healthy and vaccinated dogs showed negative reaction.

The canine distemper viral antibody was also assessed by using indirect ELISA. A total of 303 dogs including 113 CD suspected and 190 healthy and vaccinated dogs, 202 (66.66 per cent) cases having the antibody titre of 1:100 and above were considered as positive. Among 104 seropositive dogs of 113 CD suspected, the antibody titre of 1:100, 1:200, 1:400, 1:800 and 1:1600 were present in 22 (21.15 per cent), 18 (17.3 per cent), 11(10.57 per cent), 17 (16.34 per cent) and 36 (34.61 per cent) cases, respectively. In the vaccinated group containing 17 dogs, 15 (88.23 per cent) seropositive dogs had the antibody titre of 1:100, 1:200 and 1:400 in 3 (20 per cent), 5 (33.33 per cent) and 7 (46.66 per cent) cases, respectively. The healthy dogs screened for the presence of antibody against canine distemper, 83 (48 per cent) cases were positive and the antibody titre of 1:100, 1:200 and 1:400 were present in 36

(43.37 per cent), 33 (39.76 per cent) and 14 (16.86 per cent) cases, respectively.

The dogs in the age group of 0-1 year and 1-3 year were more seropositive in this study. The prevalence of canine distemper was more in male dogs; and among the breeds, in the Alsatian were more seropositive. Seasonal prevalence was higher during May, June and July followed by January and February.

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* Originals not consulted

Appendix

PROFORMA FOR COLLECTION OF DETAILS FROM THE DOG OWNER

1. Serial No. :

2. Case No. :

3. Name and address of the owner :

4. Species :

5. Breed :

6. Age & date of birth :

7. Sex :

8. Colour :

9. In case of pup, whether the dam was immunized against canine distemper :

10. History :

11. Whether vaccinated against canine distemper : Yes/No

    a. If yes,

        i) Type of vaccine used :

        ii) Age at vaccination :

        iii) Whether dewormed before vaccination :

12. Whether the animal suffered from canine distemper : Yes/No

    a. i) When did it suffer :

        ii) Name of doctor treated :

        iii) Treatment given :

        iv) Course of the disease :

SEROMONITORING AND DIAGNOSIS OF CANINE DISTEMPER

By

KANAGARAJ, C.

ABSTRACT OF A THESIS

Submitted in partial fulfilment of the requirement for the degree

MASTER OF VETERINARY SCIENCE

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DEPARTMENT OF PREVENTIVE MEDICINE

COLLEGE OF VETERINARY AND ANIMAL SCIENCES

MANNUTHY, THRISSUR, KERALA

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ABSTRACT

The prevalence of canine distemper in three hundred and three dogs from different parts of Thrissur including 113 canine distemper suspected dogs and 190 healthy and vaccinated dogs was studied.

Among 113 canine distemper suspected dogs, 11.5 per cent and 7.96 per cent had the CD viral antigen in lacrimal and nasal discharge, respectively by AGID. Counter immuno electrophoresis detected CD viral antigen in 16.81 per cent and 7.96 per cent dogs using lacrimal and nasal discharge, respectively.

Canine distemper inclusions were detected in corneal impression smears of 3.53 per cent dogs by Giemsa staining and CD viral antigens were detected in 62.83 per cent cases of suspected dogs by indirect immunoperoxidase test.

AGID and CIEP detected canine distemper viral antibody in 7.96 per cent cases of CD suspected dogs. Healthy and vaccinated dogs showed negative reaction.

Out of 303 dogs tested by indirect ELISA 66.66 per cent dogs had the protective level of antibody against CDV. Among 104 seropositive dogs of CD suspected, the antibody titres of

1:100, 1:200, 1:400, 1:800 and 1:1600 were present in 22 (21.15 per cent), 18 (17.3 per cent), 11 (10.57 per cent), 17 (16.34 per cent) and 36 (34.61 per cent) cases, respectively. In the 15 seropositive dogs which were vaccinated against CD, the antibody titres of 1:100, 1:200 and 1:400 were present in 3 (20 per cent), 5 (33.33 per cent) and 7 (46.66 per cent) cases, respectively.

The healthy dogs screened for the presence of CD viral antibody, 83 (48 per cent) cases were positive to CD and the antibody titres of 1:100, 1:200 and 1:400 were present in 36 (43.37 per cent), 33 (39.76 per cent) and 14 (16.86 per cent) cases, respectively.

More seropositive dogs were recorded in the dogs below 3 years of age and in male dogs. Among the breeds, Alsatian were more affected. Seasonal prevalence in the present study was higher during May, June and July followed by January and February.