

## Assessment of canine distemper virus infection in vaccinated and unvaccinated dogs

D Latha<sup>1</sup>, S R Srinivasan<sup>2</sup>, P S Thirunavukkarasu<sup>2</sup>, L Gunaselan<sup>2</sup>, P Ramadass<sup>3</sup> and R B Narayanan<sup>1\*</sup>

<sup>1</sup>Centre for Biotechnology, Anna University, Chennai 600 025, India

<sup>2</sup>Small Animal Clinics and <sup>3</sup>Department of Animal Biotechnology, Madras Veterinary College, Chennai 600 007, India

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Canine distemper is the most prevalent viral disease of dogs, which has high mortality rate. Vaccination of dogs against canine distemper is the only measure to control the disease. The aim of the present study was to find out the effect of commercial live attenuated vaccines in the control of distemper and the importance of the annual vaccination against distemper based on the occurrence of infection. One hundred and sixty conjunctival samples, collected from dogs with clinical symptoms suggestive of canine distemper, were screened by Dot-Enzyme Linked Immunosorbent Assay (Dot-ELISA) for the presence of canine distemper virus. The application of Dot-ELISA in screening the samples was validated using Indirect Immunofluorescence assay as the standard technique. Out of the 160 samples tested by Dot-ELISA, 112 (70%) were positive for canine distemper. The results of Dot-ELISA used for epidemiological study based on sex, age and vaccination status showed that dogs of both the sexes and 1-5 years of age were more susceptible to Canine distemper virus infection and there is a lack of regular annual vaccination of dogs which results in the high incidence of distemper in dogs of 1-5 years of age. The present study suggests that vaccination followed by regular annual boosters against distemper would effectively protect the dogs from the disease.

**Keywords:** Dot-ELISA, canine distemper, canine distemper virus, vaccination

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### Introduction

Canine distemper (CD) in dogs is caused by Canine distemper virus (CDV), a member of the genus Morbillivirus in the Paramyxoviridae family. CDV is closely related to Measles virus of human and Rinderpest virus of bovine<sup>1</sup>. CD is highly infectious and frequently a lethal disease in dogs and has a high mortality rate after Rabies. The disease is transmitted through aerosol and the virus has high affinity for lymphocytes and macrophages. The duration and severity of the disease depends mainly on the ability of the infected animal to quickly mount an immune response to CDV. If the serum antibody titre reaches high level within 8-9 d of infection, the virus disappears from the lymphatic and the other tissues and the infection remains subclinical or mild. However, if the immune response is weak or delayed the virus disseminates to many tissues causing an acute or chronic disease with high mortality<sup>2</sup>. The clinical signs may range from no visible signs to

severe disease with or without central nervous system (CNS) signs. The clinical signs are mucopurulent nasal and conjunctival discharge, biphasic fever, anorexia, depression, diarrhoea, hyperkeratosis of the footpads and the nose followed by CNS signs. There is no specific therapy for this deadly disease. The only effective approach is immunization of dogs by vaccination. Live attenuated vaccines of either avian egg origin or canine cell culture adaptations have been in use for many decades and had reduced the incidence of the disease<sup>2</sup>. Even though there are many problems associated with these live attenuated vaccines<sup>3-6</sup>, they appear to be efficient against distemper. To overcome the disadvantages associated with live attenuated vaccines, research has been focused on recombinant and DNA based vaccines expressing the surface glycoproteins of CDV, which are immunogenic<sup>7-9</sup>. A recombinant canarypox vaccine has been proved successful in protecting the dogs against distemper and has been licensed few years back for its routine use. Patronek *et al* (1995) demonstrated that the lack of vaccination against distemper was associated with several hundred-fold increase in the risk of the disease<sup>10</sup>. The annual

\*Author for correspondence:

Tel: 91-44-22350772; Fax: 91-44-22350299

E-mail: rbn@annauniv.edu

vaccination of dogs with these vaccines has been considered as the effective measure in controlling the disease.

In India, only live attenuated vaccines, either imported or local made are in use. There are no reports from here till date on the vaccination of dogs against Canine distemper and the annual vaccination pattern. The duration of post-vaccinal immunity after distemper vaccination and the required frequency of revaccinations remain controversial worldwide. Though, the vaccine manufacturers and other studies on distemper antibody titres recommend annual vaccination<sup>11,12</sup>, the actual practice among the public here is unknown. Thus, the present study was initiated to determine the occurrence of CDV infection in the vaccinated and unvaccinated dogs and to reveal the importance of annual vaccination based on this occurrence. To find out this, the incidence of the CDV infection in dogs vaccinated less than a year was compared with dogs vaccinated more than a year. The use of simplified Dot-ELISA for this investigation was validated using Indirect Immunofluorescence assay (IFA), a universally accepted technique for the diagnosis of CD.

## Materials and Methods

### Samples

One hundred and sixty conjunctival samples were collected from dogs brought to Small Animal Clinics of Madras Veterinary College Hospital, Chennai that showed suggestive symptoms of distemper. The samples were collected at room temperature. The common clinical symptoms observed were conjunctival and nasal discharge, diarrhoea, fever, skin pustules, hyperkeratosis and CNS signs. The samples used for the present study were collected from June 2002 to December 2002.

### Dot-Enzyme Linked Immunosorbent Assay (Dot-ELISA)

The conjunctival samples were screened by Dot-ELISA. Briefly, 1  $\mu$ L (~2  $\mu$ g of total protein) of conjunctival samples was coated on a nitrocellulose membrane and the membrane was allowed to dry for 10 min. Then the membrane was incubated with 5% skimmed milk powder in phosphate buffered saline (PBS, pH 7.4) for 30 min at 37°C. After each incubation step, the membrane was washed for 5-6 times with PBS. Polyclonal CDV antibody (1:1000) raised against the whole virus in rabbits was added as primary antibody and the membrane was incubated for 30 min at 37°C. The alkaline phosphatase (ALP)

conjugated goat anti-rabbit IgG (1:20,000) was added as the secondary antibody and the membrane was incubated again for 30 min at 37°C. After washing the membrane with PBS, it was developed using *p*-nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as the substrates and the appearance of brown colour was indicative of a positive reaction. The intensity of the brown colour is a semi-quantitative measure of the virus concentration in the samples.

### Indirect Immunofluorescence Assay

Indirect immunofluorescence assay (IFA) was carried out as per the method of Johnson on limited number of conjunctival samples<sup>13</sup>. Briefly, the conjunctival smears were fixed on glass slides by cold acetone and the slides were kept at 4°C for 1 h. Polyclonal CDV antibody raised in rabbits was added as the primary antibody (1:1000) and the slides were incubated at 37°C for 30 min. After washing the slides with PBS, goat anti-rabbit IgG labeled with Fluorescein iso thio cyanate (FITC; Sigma, USA) in 1:200 dilution was added and the slides were incubated at 37°C for 30 min. After washing the slides thoroughly with PBS, the final wash was given in MilliQ water. The slides were then dried and mounted using 9:1 of glycerol and PBS and observed under a Fluorescent Microscope with epi-illumination (Nikon, Japan). The Conjunctival epithelial cells showing granular greenish yellow fluorescence were taken as positive. The assay was simultaneously carried out on the same samples with normal rabbit serum as the control.

### Comparison of Dot-ELISA with IFA

The relative efficiency of Dot-ELISA was determined by using IFA as the gold standard. The results of both the assays were compared to determine the specificity and sensitivity of Dot-ELISA. The sample pairs, which were positive by both IFA and Dot-ELISA, were considered as true positives (TP), the IFA negative and Dot-ELISA positive pairs were called as false positives (FP) and the IFA positive and Dot-ELISA negative pairs were called as false negatives (FN). Specimen pairs that were negative by both the assays were true negatives (TN). The formulas applied were:

$$\text{Sensitivity} = [\text{TP}/(\text{TP}+\text{FN})] * 100$$

$$\text{Specificity} = [\text{TN}/(\text{TN}+\text{FP})] * 100$$

### Epidemiological Analysis

Age, sex, breed and vaccination history of the dogs from which conjunctival samples collected were recorded and analyzed. The dogs were divided into different categories based on the above parameters. Based on age, they were divided into 5 categories: 1 day-6 months, 7-11 months, 1-5 years, >5-10 years and above 10 years. The dogs were classified into 7 categories based on the breeds: Spitz, Non-descriptive, German Shepard, Labrador, Doberman, Lasapso and the final category consisted of breeds Boxer, Rajapalayam, Dashund, Dalmation, Terrier and Golden Retriever where the number of cases were less. In the vaccination history, name of the vaccine, type of the vaccine and the time since the last vaccination were noted. Examined dogs were divided into three categories based on their vaccination status: vaccinated, unvaccinated and unknown vaccination history. The dogs were further classified into two groups based on the time of vaccination. In group I, a dog was considered as vaccinated, only if it had been vaccinated within a year of sample collection, whereas in group II, even if the dog had been vaccinated once in its lifetime at any time more than a year also was considered as vaccinated.

### Statistical Analysis

The statistical significance in the infection rate between the group I and group II of the vaccination category was assessed by Chi-Square test.

## Results

### Screening of Conjunctival Samples by Dot-ELISA

The Dot-ELISA revealed that of the 160 conjunctival samples collected, 112 (70%) were positives and the remaining 48 (30%) were negatives (Fig. 1). Based on the intensity of the colour formation, the positive samples were referred to as positives and weak positives, where the intensity of the colour was less.

### Indirect Immunofluorescence Assay

Twenty-two conjunctival samples were taken randomly and analyzed by both IFA (Fig. 2) and Dot-ELISA. Out of the 22 samples, 15 were positives (68%) and 7 were negatives by IFA (Table 1). In Dot-ELISA, 14 samples were positives (64%) and 8 samples were negatives. The Dot-ELISA results were compared with IFA results. The number of true positives (10), true negatives (3), false positives (4) and false negatives (5) were identified and the

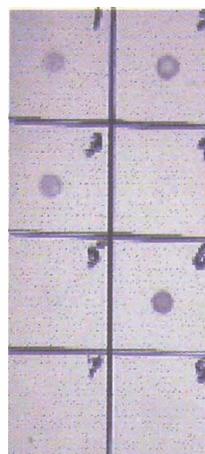


Fig. 1—Dot-ELISA for the detection of distemper viral antigen from conjunctival samples [The appearance of brown colour is an indicative of the positive reaction. Samples 1,2,3-Positives, 4, 5, 8-Weak Positives, 6-Positive control and 7-Negative control].

sensitivity and specificity of the Dot-ELISA technique was calculated using the respective formulas. Since the number of samples used for the comparison was small, the sensitivity and the specificity of Dot-ELISA were only 66% and 42%, respectively but the percent positivity was close in both of the techniques.

### Epidemiological Analysis

The number of CDV infected and uninfected dogs in different vaccination status in both the groups are shown in Fig. 3. The susceptibility to CDV infection in the group I was more in unvaccinated dogs than in vaccinated category. In group II, where the dogs had been vaccinated only once in their lifetime/more than a year back, the infection rate was the same in both vaccinated and unvaccinated.

There was no significant difference in the percentage of infection among the sexes (Fig. 4a). Age wise, dogs of 1-5 years were more susceptible to distemper infection (Fig. 5a). In the breeds, spitz was affected to a greater extent when compared to other breeds (Fig. 4b).

### Statistical Analysis

The significant difference at 5% level ( $p < 0.05$ ) was observed in the infection rate between the group I of vaccination history where the dogs had been vaccinated less than a year time and group II, where the dogs had been vaccinated more than a year back by the Chi-Square test.

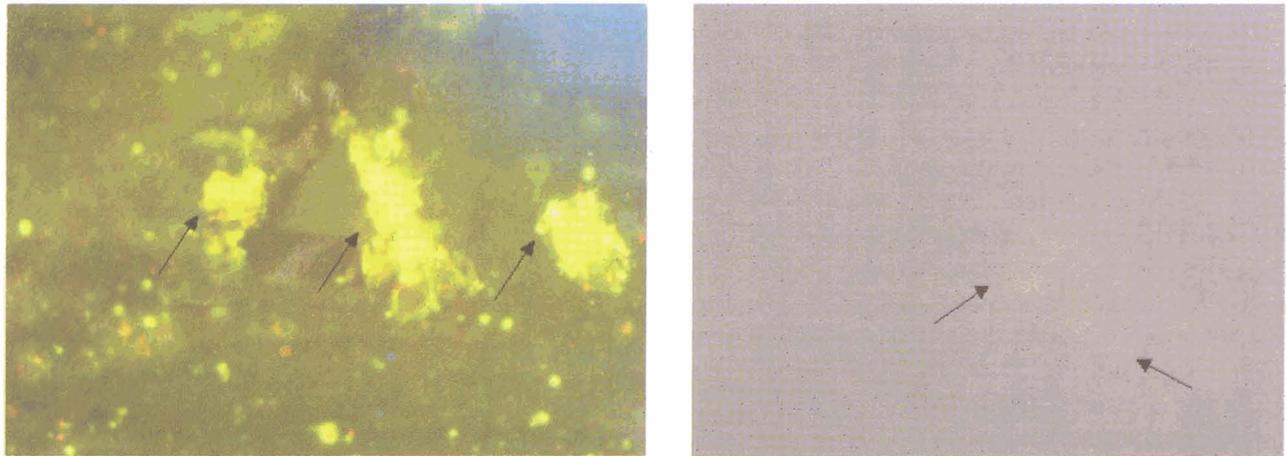


Fig. 2 (a & b)—Indirect immunofluorescence assay for the detection of distemper virus from conjunctival smears. a. Rabbit CDV polyclonal serum (X200) b. Normal rabbit serum (X200) as the control [a showing the conjunctival epithelial cells with granular greenish yellow fluorescence which represent the presence of the virus (X200) and b, the cells without any fluorescence represent no reactivity with the normal rabbit serum (X200) using Nikon fluorescent microscope].

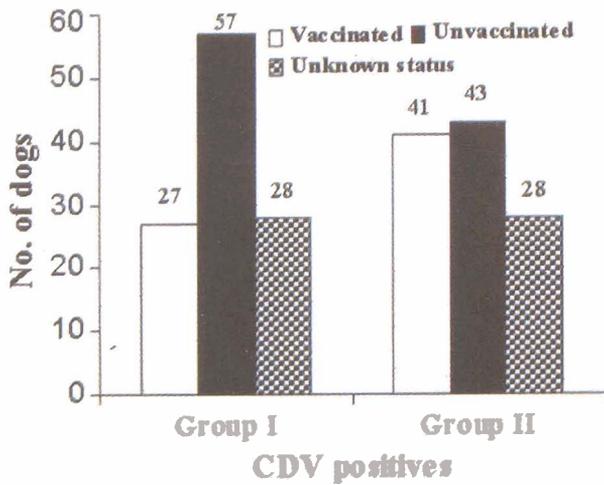


Fig. 3—Number of CDV positive dogs by Dot-ELISA in vaccinated, unvaccinated and unknown status of Group I and Group II [In Group I, dogs vaccinated < 1 year back; In Group II, dogs vaccinated > 1 year back].

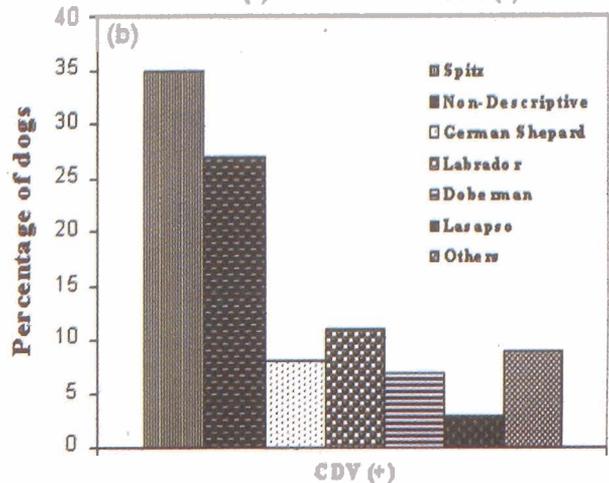
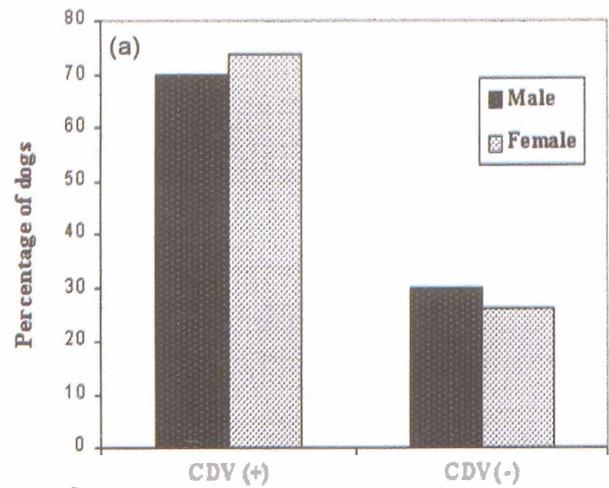


Fig. 4—CDV positivity in the conjunctival samples by Dot-ELISA, a. Male & Female dogs; b. Breeds.

Table 1—Comparison of Dot-ELISA and immunofluorescence

Dot-ELISA	IFA		Total
	Positive	Negative	
Positive	10	4	14
Negative	5	3	8
Total	15	7	22

Percentage positivity by IFA = 68%

Percentage positivity by Dot-ELISA = 64%

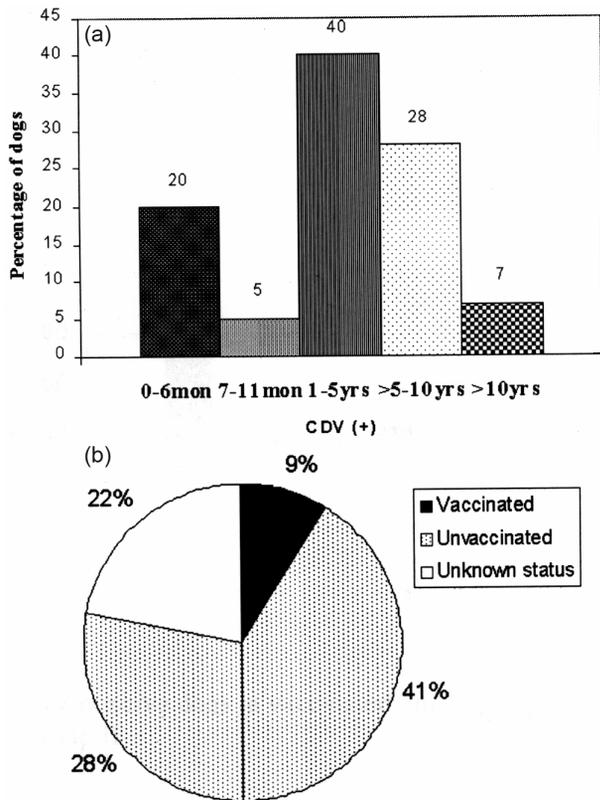


Fig. 5 (a & b)—a. CDV positivity in dogs of different age groups; b. Percentage of different vaccination status in dogs of 1-5 years of age [In the fig, both 28% and 41%, represent unvaccinated category. 28% of unvaccinated dogs lack regular annual vaccination and 41% of the dogs had never been vaccinated].

## Discussion

Canine distemper virus is resistant to cold and could survive for a long period of time under cold conditions<sup>14</sup>. So, the disease spreads mainly in the winter. This was the reason for the collection of conjunctival samples during June to December, when the reported cases of distemper will be more. The sample collection was stopped till December after getting sufficient number of samples. The dogs suspected for distemper showed various clinical signs of distemper. Neurological disorders were present both in the initial stage as well as the later manifestation after the generalized symptoms. Convulsions, paralysis and cycling (aimless wandering) were the common neurological signs observed. Conjunctival discharge was the most common clinical symptoms in the infected dogs (63%). The Dot-ELISA used to screen the conjunctival samples is simple, rapid and sensitive method for routine diagnosis of CDV infection in

dogs<sup>15</sup>. Since the percentage positivity of the conjunctival samples by Dot-ELISA (64%) was close to IFA (68%) and more number of samples could be screened within a short period of time, Dot-ELISA was used for overall screening of the collected conjunctival samples.

In the epidemiological analysis, dogs of 1-5 years of age were susceptible to CDV infection. This finding is different from the other reports where the dogs of 3-6 months of age were more susceptible<sup>16</sup>. The presence of maternally derived distemper antibodies in the young pups could be a problem at the time of primary vaccination. The average half-life of maternally derived antibodies had been calculated and the age of 3-6 months was found to be the right time to vaccinate the pups since at this age there would be complete disappearance of maternal antibodies, which will otherwise interfere with the development of vaccine antibodies. The regular practice of primary vaccination in the young pups may be the reason for the present finding that dogs of 3-6 months of age are not that susceptible compared to 1-5 years of age. The susceptibility of 1-5 years of age group to infection in this study could be because of the poor development of vaccine antibodies which may be because of the interference of maternal antibodies during primary immunization or poor storage and handling of the vaccines or immune status of the animals, which would result in quick depletion of vaccine antibodies and the lack of routine vaccination of dogs.

Analysis of vaccination history of the dogs showed that the infection rate is more in unvaccinated dogs than in vaccinated if the period after vaccination is less than a year. If the period after vaccination exceeds a year, the vaccinated dogs will also have the same risk as unvaccinated dogs to the infection. This clearly shows that there is a need for annual vaccination of dogs. Previous Studies on vaccination showed that the protective antibody titre decreased to low levels in 33% of the dogs 1 year after vaccination<sup>17</sup>. When the vaccination history of the CDV infected dogs of 1-5 years were analyzed the percentage of unvaccinated dogs which included dogs vaccinated more than a year was 69% and 28% of the dogs lacked annual vaccination which might be the reason for the high incidence of distemper in that category.

Both sexes are equally affected by CDV infection in this study and the percentage of CDV infected

cases was high in Spitz, a dolichocephalic breed. Though, dogs of all the breeds are susceptible to CDV infection, dolichocephalic breeds have high incidence when compared to brachiocephalic dogs<sup>2</sup>.

In conclusion, the age-wise and the vaccination status based prevalence of CDV infection suggests that regular annual vaccination would be efficient to protect the dogs against distemper infection and the vaccination significantly reduces the risk of the disease.

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