



Direct Fluorescent Antibody Test (DFAT) as A Rapid-Point of Care Test for the *Intra-vitam* Diagnosis of Canine Distemper

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10.18805/IJAR.B-5511

ABSTRACT

Background: Canine distemper (CD) is an important fatal viral disease of dogs worldwide. Subclinical infections are common in CD and they delay the diagnosis as well as the commencement of symptomatic treatment.

Methods: With the aim of early diagnosis, dogs which were brought to Infectious Disease Unit, Madras Veterinary College from April 2021 to November 2022 with the symptoms of CD used for the present study. Conjunctival (30), nasal (30) and genital (30) swabs were collected from 30 dogs that displayed signs of Canine Distemper, producing a total of 90 samples. All samples were subjected to both Nested Reverse Transcription Polymerase Chain Reaction (nRT-PCR) and Direct Fluorescent Antibody Test (DFAT) simultaneously.

Result: Both tests showed concurrence in diagnosing CD with 81.11% (73 out of 90 samples). The Kappa value of 0.57 revealed that there was a moderate agreement between the results of nRT-PCR and DFAT. Moreover, nRT-PCR is expensive and time consuming compared to DFAT and the latter is economical and requires only 2 hours for results. Hence, DFAT could be considered as a rapid-point of care test for initial screening purpose as well as to avoid unnecessary euthanasia of dogs with non-infectious respiratory and nervous signs.

Key words: Canine distemper, Direct fluorescent antibody test, Early diagnosis, Nested reverse transcription-PCR.

INTRODUCTION

Canine Distemper Virus (CDV) belongs to the genus Morbillivirus, family Paramyxoviridae and it causes multisystemic disease characterized by a variety of symptoms including biphasic fever, respiratory signs, enteritis, cutaneous lesions and neurologic disorders. Vaccination is the most effective and inexpensive way to prevent any viral diseases in dogs (Kant *et al.*, 2023). Even though, Canine Distemper (CD) can greatly be reduced by widespread vaccination, it continues to be an endemic disease in many countries including India. Kodi *et al.* (2020) mentioned that CDV is the one of the important causes for canine gastroenteritis. The clinical signs of CD varied depending on the age, immune status of the host as well as the virulence of the virus strain and the environmental conditions (Greene and Appel, 2012). There are two viraemic phases in CDV infection: the first phase lasts from 7 to 10 days post infection with the symptoms of fever, anorexia and mild respiratory symptoms. During the second viraemic phase, the virus got more disseminated to the gastro-intestinal tract, central nervous system and skin (Evermann and Kennedy, 2011). In CDV infections, 30% of dogs developed CNS signs, which usually occur 1 to 6 weeks after the onset of acute illness and majority experience subclinical infection, out of which few dogs had a rapidly progressive infection which led to death (Sykes, 2013). Many domestic dogs with CDV infection might be in subclinical phase with mild illness, nonspecific listlessness, partial anorexia, fever and upper respiratory tract infections (Deem *et al.*, 2000). Hence, it is necessary to diagnose the suspected CD cases with different samples regardless of the presence or absence of clinical signs. Positive cases (54.5%) were reported in asymptomatic and unvaccinated

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How to cite this article: Devi, T., Asokkumar, M., Bharathi, M.V., Ramesh, A., Tirumurugan, K.G. and Pazhanivel, N. (2025). Direct Fluorescent Antibody Test (DFAT) as A Rapid-Point of Care Test for the *Intra-vitam* Diagnosis of Canine Distemper. Indian Journal of Animal Research. 1-7. doi: 10.18805/IJAR.B-5511.

Submitted: 01-11-2024 **Accepted:** 07-04-2025 **Online:** 17-05-2025

dogs by real time quantitative PCR (Puerto *et al.*, 2010). However, the acute generalized form has a high mortality rate in domestic dogs with 14-18 days of eclipse period. Since the virus showed strong infectivity and the infection had a high mortality rate, most dog breeders suffered serious economic losses with CDV infection (Li *et al.*,

2013). Early treatment particularly before the onset of severe nervous signs may save the animal (Devi *et al.*, 2024). Hence, it is important to have a rapid, higher sensitive and also a cost-effective assay for the diagnosis of Canine Distemper in developing countries where the stray dog population is more. Such an assay will not only exclude other diagnoses and also helpful to prevent the further transmission of the disease from the infected dogs. With this objective, Direct Fluorescent Antibody Test (DFAT) was used as a rapid-point of care test for the detection CDV and the results of DFAT were compared with that of highly sensitive test, nRT-PCR.

MATERIALS AND METHODS

Study population and sample size

The present study was conducted on dogs which were brought to Infectious Disease Unit (IDU) of Madras Veterinary College Teaching Hospital (MVCTH), India with the clinical symptoms such as ocular and nasal discharge, seizure, temporal twitching, champing of jaws, muscle tremor, chorea, paddling movement of legs, paralysis of hind limbs and blindness between April 2021 and November 2022. Epithelial swabs (Conjunctival, nasal and genital swabs) were collected from the dogs and they were subjected to nRT-PCR. Considering nRT-PCR is a standard and highly sensitive test, three epithelial samples (impression smears) of 30 CD positive dogs (n=30) were simultaneously subjected to DFAT. The 30 dogs were positive by nRT-PCR with at least one of its three samples. Sample size of 30 derived by power analysis @95% confidence interval. Hence, conjunctival (30), nasal (30) and genital (30) swabs were collected from 30 dogs that producing a total of 90 samples. Results of 90 samples by both nRT-PCR and DFAT were compared. History of vaccination of 30 dogs was also collected from the owners.

Sample collection and processing for nRT-PCR

Conjunctival, nasal and genital swabs were collected in a sterile eppendorf tube with 1 ml of Phosphate Buffered Saline (PBS, pH 7.4) and stored at -20°C until processed. RNA was extracted from epithelial cells such as conjunctival, nasal and vaginal/prepuccial swabs by Trizol method (Agnihotri *et al.*, 2017) and Bio-Rad iscript cDNA synthesis kit was used for cDNA conversion. Upon RNA extraction, the reaction recipe was prepared for the total volume of 20µl and the cyclical condition followed for conversion of cDNA included priming (5 min at 25°C; reverse transcription (20 min at 46°C); and reverse transcription inactivation (1 min at 95°C). The synthesized cDNA was stored at -20°C. The nRT-PCR was performed as per the

method described by Alcalde *et al.* (2013) and the primers used in nRT-PCR is given in Table 1. Reaction volume and concentration of various components used in outer and inner nRT-PCR are given in Table 2. Steps and conditions of thermal cycling in nRT-PCR (both inner and outer) is given in Fig 1. The gel electrophoresis with 1.5% agarose in TAE buffer (1X) stained with ethidium bromide and visualized under UV light and the Puppy DP (Nobivac) vaccine was used as a positive control.

Sample collection and processing for DFAT

Ocular, nasal and genital swabs were used to prepare an impression smear separately on a clean glass slide from each swab and allowed to air dry for 5 minutes. Then smears were fixed in chilled acetone and methanol (1:1) in a coplin jar and kept at 4°C for 30 minutes. Smears were then stored at -20°C until processed (Seki *et al.*, 2003).

Then the smear was stained with anti-CDV-FITC conjugate (VMRD, Inc., Pullman, WA) and kept for incubation at 37°C for 1 hour (Kapil and Neel, 2015). After incubation, the slides were washed 3 times with PBS and then the smears were counter stained either with Evans blue or DAPI (4,6, Diamidino- 2 Phenyl Indole) and incubated at 37°C for 5 minutes. After washing, the slides were finally mounted in mounting fluid (9 :1 of glycerol and PBS respectively) as described by Latha *et al.* (2007) and examined under fluorescent microscope. Impression smear made from a brain sample of CD positive animal proved by cultural isolation was used as a positive control.

Statistical analysis

The diagnostic sensitivity and specificity were calculated as per Thrusfield (2018) and also the specimens were classified as true positive or true negative by the standard test, the nRT-PCR and the DFAT assay results were considered as false positive or false negative if they disagreed with the nested PCR results. The Kappa statistics was done with Graphpad version.5 to compare the association between the results of nRT-PCR and DFAT.

RESULTS AND DISCUSSION

Out of thirty dogs subjected to this study, the owner's data showed that twenty - one dogs were unvaccinated, eight dogs brought with the history of irregular vaccination and only one was vaccinated with routine vaccination schedule. On clinical manifestations, we could observe that ocular discharge either in serous or purulent nature in 24 dogs out of 30. Nasal discharge was observed in 10 dogs and temporal twitching and champing of jaws were noticed in nine dogs. Whereas, seven dogs could not able to walk

Table 1: Primers used in Nested RT-PCR (Alcalde *et al.*, 2013).

Gene target	Name of the primers	Sequences of primers used	Amplicon size in bp
CDV N gene	Outer forward	TAA GCT GGG TCAAAG TAA GAT CG	336
	Outer reverse	GAATTG CTG AAA TGA TTT GTG AT	
	Inner forward	TTG GCA TTG AAA CTATGT ATC C	234
	Inner reverse	CGAAAC CCAACC CTC CCATG	

and other had various combination of fever, blindness, chorea, muscle tremor, inco-ordination, diarrhoea and vomiting.

The expected 234 bp fragment of amplified CDV N gene by nRT-PCR assay in samples are shown in Fig 2. Impression smear taken from live animal subjected to DFAT after counter-stained with Evans blue is depicted in Fig 3. Smears stained with DAPI showing positive fluorescence and negative result are shown in Fig 4a and 4b respectively and the Positive control (CDV isolate) with fluorescence is depicted in Fig 5.

Table 2: Reaction volume and concentration of various components used in nRT-PCR.

Components	Volume	
	Outer	Inner
PCR master mix (2X)	10 µl	10 µl
Forward primer (10pmol/ µl)	1 µl	1 µl
Reverse primer (10pmol/ µl)	1 µl	1 µl
Template cDNA	2 µl	1.5 µl
Nuclease free water	6 µl	6.5 µl
Total volume	20 µl	20 µl

Although only 56 and 67 samples out of 90 samples tested were shown positive by nRT-PCR and DFAT respectively, both tests were yielding similar results for 73 samples (53 positive and 20 samples negative). Hence, nRT-PCR and DFAT were in concurrence in diagnosing the Canine Distemper with 81.11% (73 out of 90 samples). Moreover, kappa statistics calculated the kappa value of 0.57 between these two tests and it revealed that there was a moderate agreement between the results of RT-PCR and DFAT. The comparison of results of nRT-PCR and DFAT is depicted in Table 3. The detailed results of 90 samples by nRT-PCR and DFAT are shown in Table 4. The higher percentage of diagnostic sensitivity was obtained by nRT-PCR (94.64%) than DFAT (79.10%) whereas the higher percentage of specificity (86.95%) was obtained with DFAT compared to nRT-PCR (58.82%).

In our study group, majority (29/30) are either unvaccinated dogs or dogs with irregular vaccination. Hence, we could stress that Canine Distemper is a preventable disease with regular vaccination and Headley and Graca (2000) also recommended that the only method

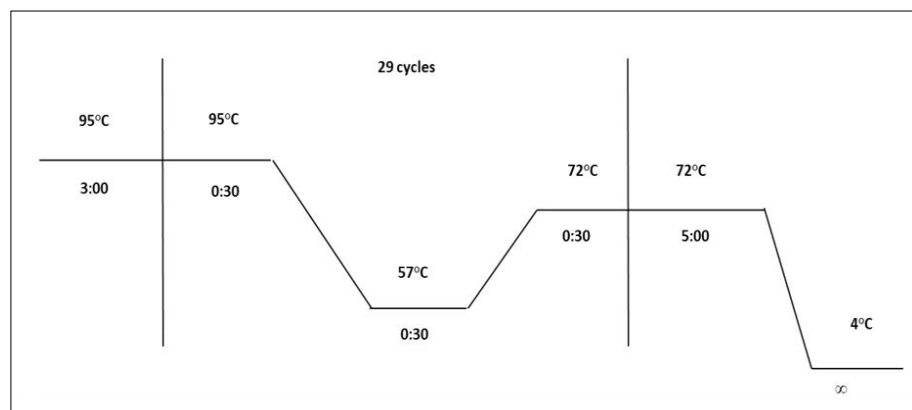


Fig 1: Steps and conditions of thermal cycling in nested RT-PCR (both inner and outer).

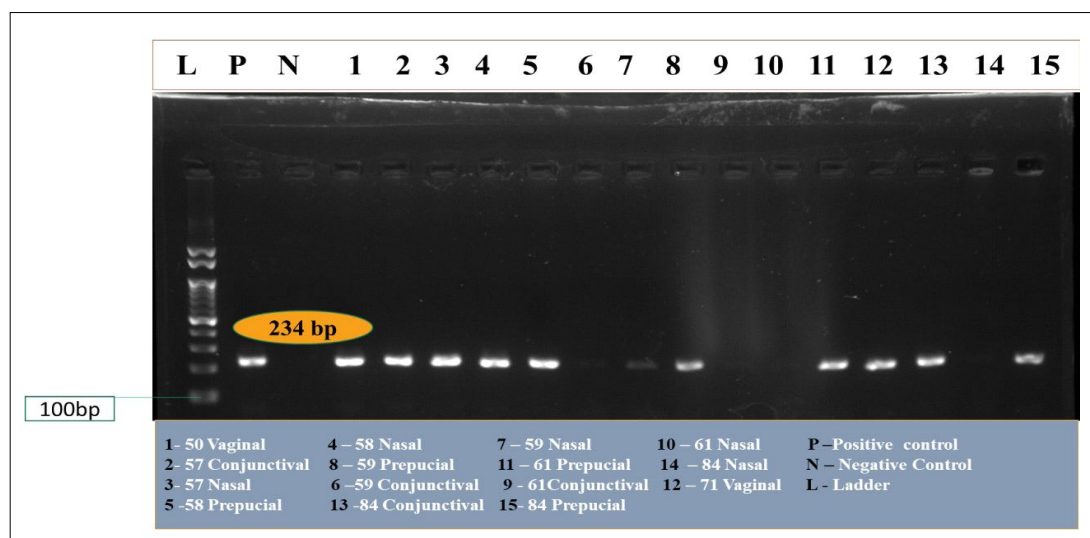


Fig 2: Nested Rt-PCR results of 15 sample (epithelial swabs) for CDV. numerical represents the sample number.

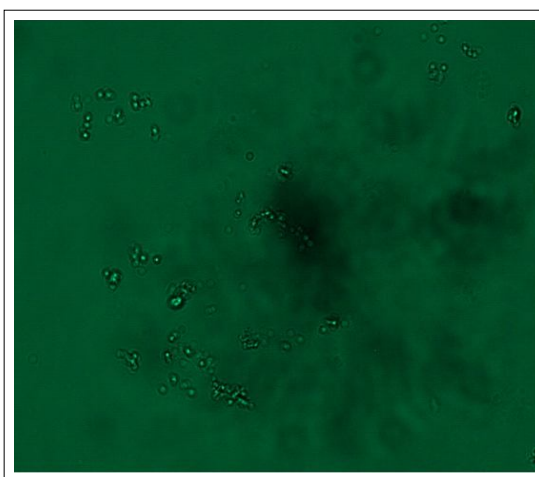


Fig 3a: DFAT- Conjunctival swab with CDV positive cells exhibiting apple green fluorescence.

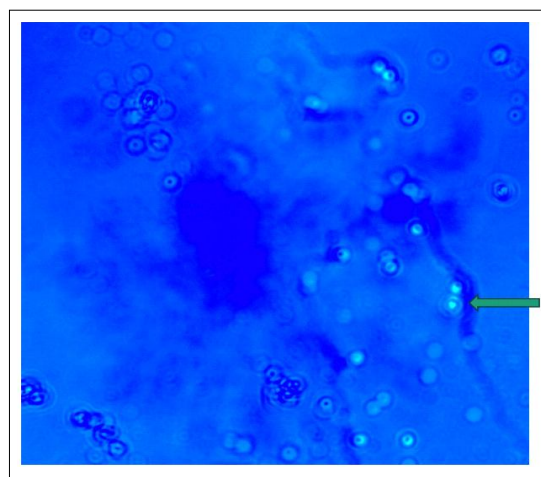


Fig 4a: DFAT- Conjunctival swab with Cdv positive cells exhibiting fluorescence after counter staining with DAPI.

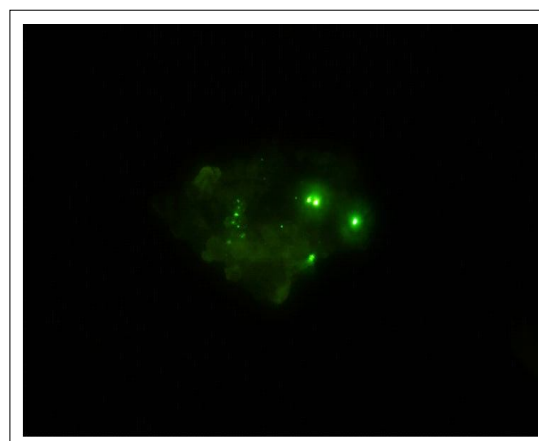


Fig 3b: DFAT- Nasal swab with CDV positive cells exhibiting apple green fluorescence and CDV-negative cell were brick red due to counterstaining with evans blue.

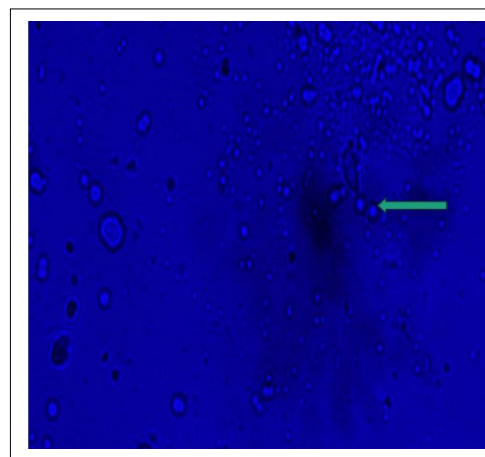


Fig 4b: DFAT-Vaginal swab with CDV negative cells showing negative result (blue) after counterstaining with DAPI.

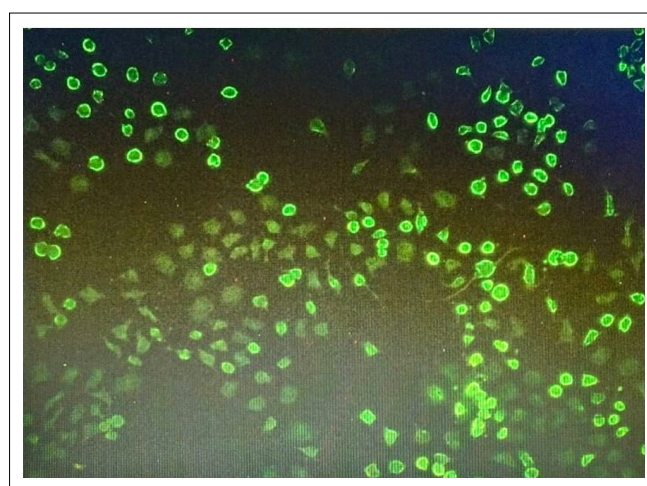


Fig 5: Positive control showing fluorescence under DFAT.

to control the occurrence of Canine Distemper was to adopt the proper immunization programme for all susceptible population. Considering the clinical signs, variability of the clinical phase of Canine Distemper might confuse its diagnosis (Saito *et al.*, 2006) and the classical symptoms cannot be expected in all the cases. Amude *et al.* (2006) also mentioned that the clinical signs could occur with various combinations which concurred with findings of the present study where various combination of clinical signs in CD infected dogs observed. Amude *et al.* (2007) found that many dogs did not exhibit classical clinical signs and infected with other pathogens which are responsible for similar signs, rendering CD diagnosis difficult.

For molecular diagnostics, we targeted Nucleoprotein (NP) gene since the amplification of CDV RNA was successfully sequenced using RT-PCR by Frisk *et al.* (1999). Shin *et al.* (2004) also opined that RT-PCR had the potential to increase the sensitivity of detecting CDV infections in dogs since NP gene plays a major role in viral

replication and moreover, the mRNA of the NP gene is transcribed in most cases of infected cells and the RT-PCR is found to be the suitable method for the ante mortem diagnosis of Canine Distemper (Saito *et al.*, 2006). Alcalde *et al.* (2013) also stated that nRT-PCR method is more effective to detect NP gene compared to single PCR. An *et al.* (2008) used the nRT-PCR as a gold standard to develop a new antemortem diagnostic test, immunochromatography (IC)-based assay for the earlier diagnosis of Canine Distemper. With this reference, present study also preferred nRT-PCR as a standard test to compare the results of Direct Fluorescent Test. An *et al.* (2008) also mentioned that Immuno-Chromatography assay had good sensitivity with conjunctival swab but not with nasal irrigation fluid and blood samples.

Since the Canine Distemper virus is pantropic, it is better to test more than one sample from the same dog to confirm the diagnosis. Silva *et al.* (2014) also agreed to the statement and mentioned that more than one type of clinical sample should be evaluated for CDV, considering the different clinical manifestations of Canine Distemper. Saliki (2020) pointed out that nasal swab might be useful for the Canine Distemper viral isolation as well for the PCR testing and also stressed that EDTA-blood is often an excellent sample for distemper diagnosis. Elia *et al.* (2006) found that conjunctival swab and urine are ideal clinical samples for detection of CDV by quantitative real-time PCR (qRT-PCR).

Shin *et al.* (2004) amplified NP gene in 19 out of 27 nasal samples from dogs which were clinically suspected for CDV. Silva *et al.* (2014) found CDV NP gene with the expected 287 bp fragment in 36.6% of urine samples Whereas Kim *et al.* (2001) detected CDV NP gene from 4

Table 3: Comparison of nRT-PCR and DFAT.

Name of the test	nRT-PCR positive	nRT-PCR negative	Total
DFAT positive	53	14	67
DFAT negative	3	20	23
Total	56	34	90

Observed percentage of agreement is = $53 + 20 / 90 = 81.11$.

Sensitivity of nRT-PCR = $53/56 = 94.64\%$.

Sensitivity of DFAT = $53/67 = 79.10\%$.

Specificity of nRT-PCR = $20/34 = 58.82\%$.

Specificity of DFAT = $20/23 = 86.95\%$.

Table 4: Detailed results of 90 samples by nRT-PCR and DFAT.

Sample number of dogs	Number of samples	Number of concurrences	nRT-PCR results			DFAT Results		
			Conj.	Nasal	Genital	Conj.	Nasal	Genital
44,87,97,102,123	15(5*3)	15	a	a	a	a	a	a
57,77,90	9 (3*3)	9	a	x	x	a	x	x
58,71,50,106	12 (4*3)	12	x	a	a	x	a	a
78,99,124,185	12 (4*3)	12	a	x	a	a	x	a
116	3	3	x	x	x	x	x	x
84,103	6	4	a	x	a	a	a	a
51,54	6 (2*3)	0	x	x	x	a	a	a
91	3	2	x	x	a	a	x	a
52	3	2	a	a	a	a	a	x
55	3	2	x	a	a	a	a	a
61	3	1	a	x	a	x	a	a
59	3	2	a	x	x	a	x	a
60	3	2	a	a	a	x	a	a
62	3	3	a	a	x	a	a	x
81	3	2	a	x	a	a	a	a
82	3	2	a	a	x	a	a	a
Total	90	73						

Note: 'a' indicates positive result and 'x' indicates negative result. green denotes concurrence, red denotes difference.

out of 5 vaccinated dogs with combined nested PCR on 2nd and 7th day of post vaccination in ocular, nasal discharge, feces, saliva and urine. But none of the samples could be amplified to get specific band after 14 days post vaccination. Kim *et al.* (2001) also obtained negative nested PCR results in Peripheral Blood Mononuclear Cells (PBMCs) samples of cases (12%) with typical clinical signs of CD. Frisk *et al.* (1999) opined that negative RT-PCR results might be due to a complete lack of CDV RNA or presence of only low levels of CDV in the samples. Jozwik and Frymus (2005) detected 52% positivity in nested PCR and 26% in DFAT out of 23 samples from dogs. Alcalde *et al.* (2013) detected NP gene of CDV with a higher frequency of positivity (44%) in urine samples, followed by PBMCs (38%) and saliva (12%). Ashmi *et al.* (2017) reported that out of the 90 samples collected from CDV suspected dogs, 21 samples (23%) were positive by nRT-PCR but our study found higher positivity of nRT-PCR with 94.64%. compared to the findings of Kim *et al.* (2001), Jozwik and Frymus (2005), Alcalde *et al.* (2013), Silva *et al.* (2014) and Ashmi *et al.* (2017). Yilmaz *et al.* (2022) opined that necropsy samples were valuable diagnostic material and nasal swabs had the highest diagnostic value in the detection of CDV with RT-PCR.

Frisk *et al.* (1999) compared the sensitivity of RT-PCR with immunohistochemical methods using different samples like whole blood, cerebrospinal fluid and tissue specimen from the CD suspected dogs and the sensitivity was found to be 86-88%. Schumaker (2012) stated that DFAT had the advantage of having the little risk of false-positive results in recently vaccinated puppies. But in the present study, the chances of false positivity are very less since 21 dogs out of 30 were not vaccinated and eight dogs were given irregularly vaccination. Only one out of 30 was vaccinated with regular schedule and the last vaccine was done 3 months back.

Kapil and Neel (2015) evaluated the usefulness of DFAT targeting CDV proteins of replicating virus in the external epithelia in field settings and also reported that DFAT on external epithelial surfaces was a prognostic tool for CDV diagnosis. In contrast, Maes *et al.* (2003) opined that the results of DFAT could not be definite due to the intensity of the fluorescence was weak.

Out of 57 dogs tested, 19 were PCR positive and 15 were positive in DFAT, whereas 37 dogs were negative in both the methods (Athanasίου *et al.*, 2017). In Present study 73 out of 90 samples had given similar results by both nRT-PCR and DFAT. Kapil and Neel (2015) reported that the analytical sensitivity of the DFAT was lower than that of the real-time RT-PCR assay for CDV but also added that application of the CDV FAT was useful to avoid false-positive results due to recent vaccination. Though Immuno fluorescence (IF) had less sensitivity for the detection of CDV, It had the advantage of being fast and unexpensive (Schumaker *et al.*, 2012). Del Puerto *et al.* (2010) also opined that DFAT had low sensitivity and could generate

false negative diagnoses. On the contrary Athanasίου *et al.* (2017) stated that FAT was 100% specific and an adequately sensitive assay (sensitivity: 78.95%) for antemortem diagnosis of Canine Distemper. In our study also, higher percentage of specificity (86.95%) was obtained in DFAT compared to nRT-PCR (58.82%).

Importantly, only 2 hours are needed to complete DFAT compared to nRT-PCR which requires a minimum of 8 to 10 hrs. Upon comparison with nRT-PCR, the DFAT is much cost effective, less time consuming and versatile technique if fluorescent microscope is available to expedite the diagnosis of CD. Moreover, nRT-PCR and DFAT were in concurrence with good options for diagnosing the CD with 81.11% (73 out of 90 samples). Kappa value of 0.57 between these two tests revealed that there was a moderate agreement between the results of nRT-PCR and DFAT.

CONCLUSION

DFAT could be used as rapid-point of care test for initial screening of Canine Distemper on account of commencement of early therapeutic management. With a cost-effective rapid-point of care test, Canine Distemper can be quickly ruled out since the DFAT had shown high specificity and positive predictive value. The higher specificity of DFAT aids in avoiding unnecessary euthanasia of dogs with non-infectious respiratory and nervous signs.

ACKNOWLEDGEMENT

The authors are grateful to Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), for providing funds for the present work. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors declare that they have no conflict of interest.

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