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Optimization of competitive lateral flow assay for detection of canine distemper virus antibody

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Abstract
Canine distemper (CD) is a highly contagious and invariably fatal disease of domestic dogs and other carnivores having the second highest mortality rate in dogs, after rabies. The number of CDV outbreaks in vaccinated dogs and wildlife has increased in past, which demands the effective disease control strategy. The present study was designed to assess the potential of antibody detection in competitive mode by the LFA. Prior to testing serum samples, they are incubated with a fixed quantity of CDV antigen. The complete neutralization of antigen by the presence of antibody in the serum sample results in no binding at the test line. The intensity of the red band in the test line is inversely proportional to the concentration of antibodies in serum samples. The titre values of serum samples were primarily determined by virus neutralization test (VNT). Based on its titre values, the serum samples having titre up to 1:32 were found to be detected by LFA. The related viruses like PPRV, Measles, Canine Adenovirus, and Parvovirus serum samples did not show any cross-reactivity. Therefore, the optimized mAb-based LFA can be used as an on-site diagnostic tool for antibody detection in dogs as well as wildlife species.

Keywords: Lateral flow assay, competitive mode, canine distemper virus, antibodies, monoclonal antibodies, nucleoprotein

Introduction
Canine Distemper is caused by Canine morbillivirus, a monopartite, and negative sense virus of *Morbillivirus* genera of the family *Paramyxoviridae* (ICTV 2018b) [6]. There is only one serotype of CDV, with various genetic lineages (genotypes) based on the level of amino acid diversity in the Haemagglutinin (H) gene (Panzera *et al*., 2011) [14]. Strains having amino acid divergence of less than 3.5-4% falls under the same lineage and more than 4% belong to different lineages (Martella *et al*., 2006; Loot *et al*., 2018) [8, 7]. Currently identified lineages are America 1 to 5, Europe1/South America1, South America 2, South America 3, Arctic-like, Rockborne-like, Asia 1 to 4, Africa 1 and Africa 2, European Wildlife (Martella *et al*., 2008; An *et al*., 2008) [9]. India-1/Asia-5 was identified and reported by our laboratory in 2018 (Bhatt *et al*., 2018) [4]. The South America/North America – 4 and Asia-6 lineage from China has been reported recently stating the continuous emergence and intercontinental spread (Rendon-marin *et al*., 2020; Wang *et al*., 2021) [15, 21].

The virus infects the respiratory, gastrointestinal, and central nervous systems resulting to various clinical signs like sneezing, coughing, fever, lethargy, hyperkeratosis of the foot-pad and nose. It mainly affects dogs (*Canis familiaris*) of all ages and is also reported to affect many wild animals. Although a very little is known about the transmission and maintenance of the virus in wildlife, the virus has been constantly reported in various species of Canids, Hyaenids (Loots *et al*., 2018) [7], Felids (Mourya *et al*., 2019) [10], Mustelids, Procyonids, Ursids, Viverrids, and some non-human primates (Beineki *et al*., 2009). Cheetah, domestic cats, and Asian Elephants have been reported serologically positive for CD without any clinical signs (Munson *et al*., 2004) [11]. The increasing reports of CDV in wildlife animal species demand a rapid on-site assay for antibody detection in dogs as well as wildlife animals.

Sero-conversion in CDV infected dogs is observed in 2-3 weeks with a high titre of neutralizing antibodies (Appel MJ, 1969) [2]. The virus neutralization test is the gold standard test for CDV antibody detection. The cell culture handling skills and the time required by VNT presents its disadvantages. ELISA is also a highly specific and sensitive technique but requires a minimum of 2-3 hours and laboratory backing.

It is widely used to detect CDV antibodies in both dogs and non-dog hosts (Greene *et al*., 1984) [5] and gives similar results to the serum neutralization test (SNT) (Noon *et al*., 1980) [12]. The plaque reduction neutralization tests, immunoperoxidase plaque staining for CDV detection are some specific and sensitive tests for serological study (Oyedele *et al*., 2004; Soma *et al*., 2001) [13, 17].

Field diagnostic tests are the need of the hour to detect the infection on-site and control the spread of disease. The lateral flow assay (LFA) holds many advantages over other tests. The requirement of a few cost-effective reagents with long shelf life, ease of performing the test, and the rapidity of the test, has increased its significance in the current scenario. Our laboratory developed an LFA (sandwich format) for CDV antigen detection using the in-house developed monoclonal antibodies against virus isolate which represent the circulating strains of India. The same strip was evaluated for the detection of CDV antibodies in competitive mode.

Material and Methods

LFA strip assembly

The LFA strip was developed using an in-house developed monoclonal antibody against nucleocapsid protein of CDV. The virus used in the study was isolated by our laboratory and characterized towards development of vaccine candidate (Indian Patent application No. 202011057169). The strip was spotted with purified mAb as capture antibody (test line) and Anti-mouse IgG (Whole Molecule) (Sigma M7023) as the control line. The same mAb was used for bioconjugate preparation. The distance of the test line was optimized at 0.8 cm from the conjugate pad and 1cm to the control line (Figure. 1). The laminate was left to dry for 1-2 hrs at room temperature, the strips were cut manually (5mm each) and stored at 4 °C until use.

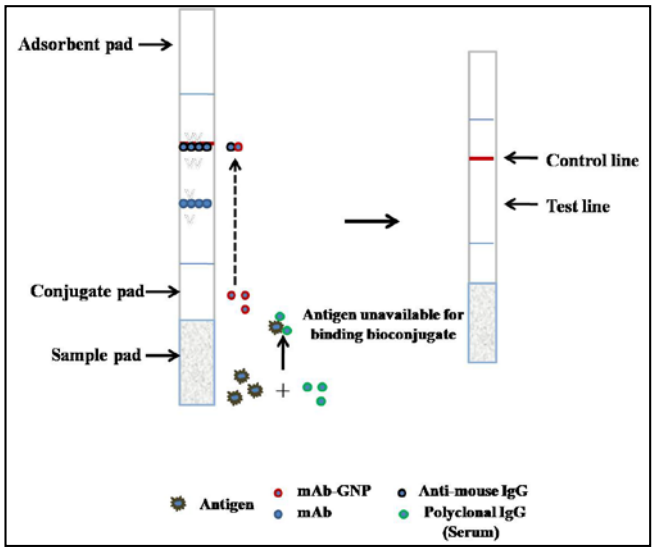

Fig 1: Schematic representation of antibody detection in CDV positive serum samples by competitive mode LFA

Preparation of anti-CDV mAb-conjugated GNPs (Detecting antibody/Bioconjugate)

Conjugation of mAb with gold nanoparticles (GNPs) was done by mixing 20 µg/mL mAb with 5 mM KH2PO4 buffer (pH 7.5) and 900 µL of 30 nm GNPs, followed by incubation at room temperature for 30 minutes. 100 µL of 10% BSA in 50 mM KH2PO4 (pH 9.0) and 1% PEG-8000 in 50 mM KH2PO4 (pH 7.5) were added to the solution and again by incubation at room temperature for 30 minutes. The incubated solution was centrifuged at 14,000 rpm at 4 °C for 25 minutes and the pellet was dissolved by sonication. 1 ml of preservation solution (20 mM Tris HCl, 0.1% Bovine serum albumin (BSA), 0.01% sodium azide, 0.05% PEG-20,000, pH 8.2) was added to the dissolved pellet and incubated again at room temperature for 1 hour. The suspension was subjected to final centrifugation and the supernatant was discarded leaving 20-30 µL of remaining preservation solution. The final pellet was dissolved by sonication to get the bioconjugate and kept at 4 ℃ for use.

Determination of minimum antigen quantity required for blocking

The highest dilution of ultra-purified CDV antigen ($10^{8.5}$ TCID50/ml) that is distinctly detected in the LFA strip was selected for blocking. Two-fold dilution of CDV positive serum (1:1024 VNT titre value) was added to an equal volume of ultra-purified CDV antigen dilution and incubated for 1 hour at 37 ℃. The LFA strip designed for antigen detection in sandwich format (unpublished data) was used for anti-CDV antibody detection in a blocking mode following the principle of competitive ELISA (Singh *et al*., 2004) [16]. The incubated mixture of antigen and serum was tested in LFA strip.

Optimization of sample running buffer

The components of sample running buffer are aimed to keep at minimum level. Primarily, Phosphate-buffered saline (PBS), pH 7.5 was screened as sample running buffer. To provide optimum movement of sample, clearance of membrane and avoid non-specificity, Tween- 20/80 and Triton-X 100 were added at different concentrations (0.5%, 0.2%, 1%, 2%, 4% and 5%). Also, the pH range of the optimized buffer (7.0, 7.5, 8.0, 8.5, and 9.0) was screened.

Analytical performance characteristics

Two-fold dilution of a CDV positive serum sample (1:1024 VNT titre) was used to determine the detection limit of the assay. And the analytical specificity was determined by testing known positive serum samples of the related viruses of same genera (PPRV and Measles) and other common viruses of the dog (Canine parvovirus and Canine adenovirus).

Diagnostic performance characteristics

Serum samples of CDV were incubated with ultra-purified CDV antigen at room temperature for 1 hr. Positive serum neutralizes the antigen and makes it unavailable for binding at the test line giving only one band at the control line (Figure 2). Negative serum samples leave the antigen available for binding the bioconjugate and mAb at the test line giving two red bands. The serum samples, pre-incubated with antigen diluted in sample running buffer were tested by the LFA strip. The results were qualitatively analyzed based on the absence and presence of a red band in the test line as positive and negative, respectively.

The results of LFA were compared with the virus neutralization test, the gold standard test for antibody detection (Von Messling *et al*., 1999). Different sets of CDV positive sera samples were obtained from our laboratory repository. Few serum samples from wildlife were also included in the study.

The correlation between LFA and VNT for determining the end-point antibody titre was established using a known CDV

positive serum from vaccinated dogs. The relative diagnostic sensitivity (Dsn) and specificity (Dsp) of known CDV positive and negative samples were calculated using a two-sided contingency table (Singh *et al*., 2004) [16]. Positive predictive values (PPV) and negative predictive values (NPV) were also estimated from the generated data. The agreement between the LFA and VNT was determined by calculating the quotient of agreement (kappa).

Results

LFA assembly and bioconjugate preparation

The test strip was assembled using its different components as shown in Figure 1. The laminates were spotted with optimized concentrations of the mAb against CDV-N protein at the test line and Anti-mouse conjugate at the control line. The minimum concentration of 15 µg mAb was found to stabilize GNPs and form stable bioconjugate. The optimum volume of bioconjugate was found to be 2 µl, as it shows clear intense red bands in the control line without any smearing of NCM. The optimum assay run time was 8-10 min.

Determination of minimum antigen quantity required for blocking

The minimum quantity of antigen for blocking was estimated by LFA. Two fold dilution of ultra-purified CDV antigen shows the detection up to 1:10 dilutions ($10^{7.5}$ $TCID_{50}$/ml). The CDV antigen diluted in sample running buffer (PBS + x % Triton-x) was mixed with serum in 1:1 dilution and kept at 37 ℃ for 1 hr for neutralization of available antibody in serum. The dilution at which no antigen was available to bind at the test line indicated complete neutralization of antigen and only control line appeared. Whereas in case of negative samples both test and control line appears (Figure 2).

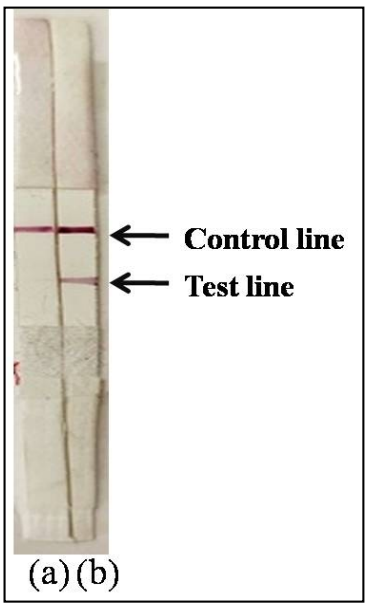

Fig 2: Detection of CDV antibodies (a) Positive serum sample (b) Negative serum sample

Optimization of sample running buffer

1M PBS, pH 7.5 showed non-specific binding at the test line as sample running buffer. Therefore the salt concentration was reduced to 0.1 mM to reduce the non-specificity. Among the different concentrations of Triton-X and Tween-20 and pH of buffer, Triton-x at 2%, pH 7.5 was found to be suitable for antibody detection.

Analytical performance characteristics

Two-fold dilution of CDV serum sample (1:1024 VNT titre) was tested to determine the analytical sensitivity of the assay. The complete neutralization was seen up to 1:16 dilution of the CDV serum, indicating the detection limit of the assay up to 1:32 titre value (Figure 3a).

No cross-reactivity was found with the positive serum samples of PPRV, Measles, Parvovirus, and adenovirus, as the red band at test line appeared in all the samples except CDV (Figure 3b).

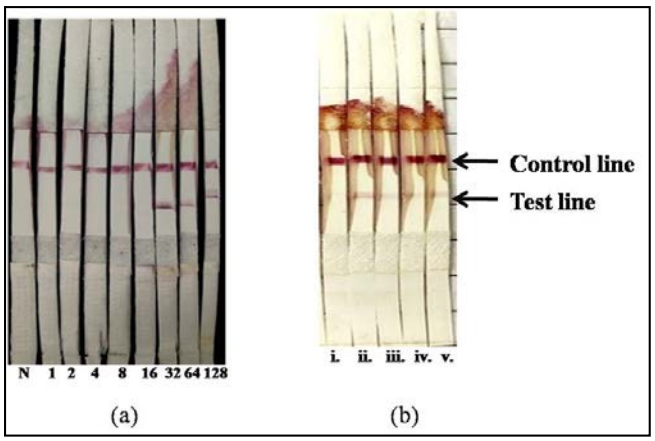

Fig 3: Analytical sensitivity (a) and specificity detection (b) of CDV antibody by LFA in competitive mode

Diagnostic performance characteristics

The serum samples were primarily screened by VNT and based on its titre values; the positive and negative samples were defined. The paired serum samples of experimentally infected dogs from zero day to 55th day post-infection were screened available in laboratory repository. The serum samples showed consistent results with VNT and samples above 1:32 titre were distinctly detected by LFA (Figure 4).

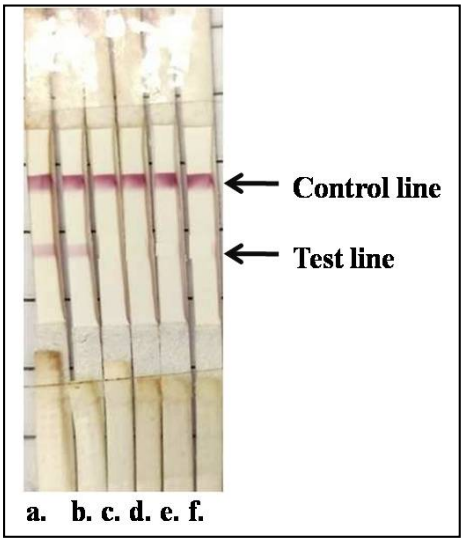

Fig 4: Detection of CDV antibody in paired serum samples of experimentally infected animal

Table 1: Details of the CDV serum samples screened by VNT and LFA

S.no.	Sample ID	Species	VNT titre*	LFA result
1.	Animal 1 – 0 dpi	Dog	0	-
2.	Animal 1– 7 dpi	Dog	1:16	-
3.	Animal 1– 14 dpi	Dog	1:4096	+
4.	Animal 1 – 28 dpi	Dog	1:8192	+
5.	Animal 1– 35 dpi	Dog	1:4096	+
6.	Animal 1– 57 dpi	Dog	1:1024	+
7.	Animal 2 – 0 dpi	Dog	0	-
8.	Animal 2 – 7 dpi	Dog	1:128	-
9.	Animal 2– 14 dpi	Dog	1:2048	+
10	Animal 2– 28 dpi	Dog	1:2048	+
11.	Animal 2– 35 dpi	Dog	1:2048	+
12.	Animal 2 – 57 dpi	Dog	1:512	+
13.	Animal 3 – 0 dpi	Dog	Less than 1:4	-
14.	Animal 3 – 5 dpi	Dog	Less than 1:4	-
15.	Animal 3– 10 dpi	Dog	1:64	+
16.	Animal 3– 17 dpi	Dog	1:256	+
17.	Animal 3– 25 dpi	Dog	1:2048	+
18.	SI-1473	Lion	>1:128	+
19.	SI-1474	Lion	>1:128	-
20.	SI-1490	Leopard	<1:8	-
21.	SI-1489	Leopard	>1:128	+
22.	SI-1497	Lion	>1:128	-
23.	SI-1496	Lion	>1:128	+
24.	SI-1498	Lion	>1:128	+
25.	SI-1499	Lion	>1:32	+
26.	SI-1500	Lion	<1:8	-
27.	SI-1505	Lion	>1:128	-
28.	SI-1473	Lion	>1:128	+
29.	SI-1489	Leopard	>1:128	+
30.	SI-1496	Lion	>1:128	+

VNT titre* - Titre above 1:32 were considered positive
- Indicates negative result
+ indicates positive result

The results of LFA and VNT were compared by a two-sided contingency table to calculate diagnostic efficacy (Table 2.). The relative diagnostic sensitivity and specificity of LFA for antibody detection are 88.4% and 100%, respectively. The positive predictive value (PPV) and negative predictive value (NPV) are 100% and 70%, respectively. A substantial agreement between LFA and VNT was observed as reflected by the kappa value of 0.765 with 95% confidence interval.

Discussion

Canine distemper virus (CDV) is a highly contagious pathogen that causes canine distemper (CD) in dogs and many other wild animal species. It causes generalized infection involving respiratory, nervous, and gastrointestinal signs in dogs and non-dog hosts. The correct diagnosis is an indispensable component of disease control strategies. VNT and ELISA are being routinely used for CDV antibodies detection. Both the tests are highly sensitive, and require sophisticated laboratory equipment and skills to perform. For canine distemper virus, the on-site diagnostic tests to detect currently circulating strains are the need of the hour. The on-site diagnostic tests are helpful in diagnosing a disease at an early stage and control its spread, as needed in case of particularly CDV to prevent its continuous spill over to wildlife species. The suitability of LFAs to detect the analyte rapidly and the ease to perform the test shows its advantages over another diagnostic test.

The present study included the LFA developed for antigen detection using one mAb both as capture and detecting reagent in a sandwich format. The strip was evaluated for the detection of CDV antibodies in competitive mode. The minimum amount of CDV antigen detected by LFA was selected for neutralization of antibodies in serum. Serum samples were incubated with CDV antigen ($10^{7.5}$ $TCID_{50}$/ml) for 1 hour at 37 ℃ for inactivation, as done in the serum neutralization test. This mixture was tested in the LFA strip, the red band in the test line indicated the negative serum sample and no band indicated positive serum samples. The serum samples of infected and vaccinated animals were tested and the assay was able to differentiate between positive and negative serum samples. The intensity of the test line is found to be inversely proportional to the concentration of antibodies in serum samples, indicating the working efficiency of the assay. The VNT was used as a definitive indicator or gold standard test for determining the diagnostic sensitivity, diagnostic specificity, positive and negative predictive values for the developed assay (Trevethan, R. 2017) [19]. Based on the VNT titre values, serum samples having titre 1:32 or above were clearly detected by the LFA. Serum titre is the value indicating the dilution up to which it can neutralize the virus. The serum sample showing positive result by LFA can give an idea of protective antibody titre in affected animal, as it is reported to be 1:32 (Taguchi *et al.*, 2011) [18]. The assay was found to be specific for CDV serum samples, as no cross-reactivity was seen with PPRV, Measles, CAV, and CPV. The Dsn and Dsp were found to be 88.4% and 100%, respectively. This preliminary study was done with a small sample size, therefore to validate the assay large number of serum samples need to be screened.

Conclusion

The optimized LFA can be used for screening the CDV suspected serum samples of the dog as well as wildlife animals in field conditions. The comparable diagnostic efficacy of the assay and easy use as compared to VNT indicates its suitability as a rapid and specific diagnostic test. Also, the assay presents an advantage over ELISA and VNT when high throughput sampling is not required and only a single sample needs to be screened.

Declaration

Acknowledgement

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Conflict of interest

The authors declared no potential conflicts of interest concerning research, authorship, and/or publication of this article.

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