Canine Distemper Virus Antigen Detection in External Epithelia of Recently Vaccinated, Sick Dogs by Fluorescence Microscopy Is a Valuable Prognostic Indicator

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Currently, there are no reliable predictors of the clinical outcomes of domesticated dogs that have been recently vaccinated against canine distemper virus (CDV) and develop respiratory disease. In this study, vaccinated dogs from Oklahoma City that were showing clinical signs of respiratory disease were evaluated for CDV antigen using a direct fluorescent antibody test (FAT). Clinical outcomes after standard symptomatic therapy for respiratory disease were recorded, and a statistical analysis of the results was performed. We present our study showing that CDV FAT results were predictive of clinical recovery (prognostic indicator, prospects of clinical recovery) among vaccinated dogs showing clinical signs of respiratory disease. Negative CDV FAT results equated to 80% chances of recovery after symptomatic therapy, compared to 55% chances of recovery when the CDV FAT results were positive. Based on the results of this study, we show that veterinarians can make better informed decisions about the clinical outcomes of suspected CDV cases, with 2-h turnaround times, by using the CDV FAT. Thus, antemortem examination with the CDV FAT on external epithelia of recently vaccinated, sick dogs is a clinically useful diagnostic test and valuable prognostic indicator for veterinarians. Application of the CDV FAT to these samples avoids unnecessary euthanasia of dogs with suspected CDV.

Canine distemper virus (CDV), one of the most dreaded and often fatal viral diseases of dogs worldwide, commonly leads to mass euthanasia of dogs housed in groups. Due to the contagious nature of virus-laden aerosols, shelters have been depopulated to contain outbreaks of canine infectious respiratory disease complex (CIRDC). In spite of the availability of commercial vaccines that can provide protection, the virus continues to circulate in the United States, due to insufficient population immunity, contact with urban wildlife reservoirs (raccoons), group housing, and occasional CDV vaccine failures due to incomplete vaccination (1). Most commercially available CDV vaccines in the United States are genetically similar (Onderstepoort strain, America-1 genetic lineage) (1). Many other disease conditions attributable to other pathogens (bacteria and viruses) can cause similar symptoms, including upper respiratory tract disease, nasal discharge, and coughing in shelter dogs (i.e., canine infectious respiratory disease complex [CIRDC]) (2). The most common causes of CIRDC are *Bordetella bronchiseptica*, *Streptococcus zooepidemicus*, canine influenza virus (H3N8), canine herpesvirus, canine distemper virus, canine adenovirus 2, and canine parainfluenza virus 2 (3). There are no published studies of predictors of the clinical outcomes of suspected CDV cases (prognostic indicators).

Specific testing protocols and prognostic indicators for CDV, with desirable accuracy and speed, are required because owners and veterinarians often elect euthanasia of dogs with positive real-time reverse transcription (RT)-PCR results for CDV, due to fear of spread of the CDV infection. Currently, commercial diagnostic companies offer quantitative RT-PCR testing for CDV with results in 1 to 3 days. The CDV RNA copy numbers in samples from dogs depend on the sample type and the stage of CDV infection. Often veterinarians suspect CDV and submit samples for CDV testing for dogs with respiratory disease; if the dog has been vaccinated recently, this could lead to false-positive results due to

detection of modified live vaccine CDV RNA (4). Due to the extreme sensitivity of real-time RT-PCR testing for CDV, recent vaccination with modified live vaccines further complicates decisions by shelter managers to pursue symptomatic treatment or to elect euthanasia.

We evaluated a CDV fluorescent antibody test (FAT) using a commercially available polyclonal anti-CDV antibody-fluorescein isothiocyanate (FITC) conjugate (VMRD, Inc., Pullman, WA) to detect productive CDV replication by testing for CDV antigen in the cytoplasm of external epithelia (conjunctival, nasal, and genital epithelia). Replication of CDV in external epithelial surfaces is an essential initial step for productive CDV infection (5). The CDV FAT targets CDV proteins and the CDV PCR assay targets a high-copy-number matrix gene that is conserved among CDV genotypes (1). External mucosa offers easier access to potential highly cellular samples, for evaluation of the CDV infection status of dogs. The analytical sensitivity of the direct CDV FAT is lower than that of the real-time RT-PCR assay for CDV; however, application of the CDV FAT to avoid false-positive results due to

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recent vaccination with attenuated CDV needs to be evaluated and validated under field conditions for dogs with respiratory disease. Respiratory disease is the most common complaint in the early stages of suspected CDV cases. Due to the frequent election of euthanasia in suspected CDV cases, evaluation of the prognostic value of the CDV FAT (a test that checks for replicating CDV) for dogs with respiratory disease after standard symptomatic treatment of vaccinated, sick dogs is urgently needed.

To correctly evaluate the direct FAT protocol for diagnostic accuracy, we applied the Standards for the Reporting of Diagnostic Accuracy Studies (STARD) checklist of 25 items. The aim of the study was to evaluate the usefulness of direct fluorescence microscopy targeting CDV proteins of replicating virus in the external epithelia in field settings. We compared the two potential clinical outcomes of the test, i.e., CDV FAT positive (scored based on the percentage CDV antigen-positive cells) and CDV FAT negative. Veterinarians normally do not submit samples from clinically normal dogs to the Oklahoma Animal Disease Diagnostic Laboratory (OADDL) for the CDV FAT unless testing is specifically requested by the laboratory. The population under study included canine clients from a well-managed clinic with complete records on vaccination, history of respiratory sickness, and clinical outcomes after symptomatic therapy for canine respiratory disease. CDV was suspected for these dogs, and they had recently received a modified live virus vaccination for CDV as one of the components of the vaccine used at the shelter. The participants were physically examined at the Neel Veterinary Clinic (Oklahoma City, OK). The initial clinical presentation of the dogs was recorded, and then direct fluorescent antibody testing for CDV was performed. The final clinical outcomes (recovery) after treatment were recorded. The direct CDV FAT results were reported in writing within a few hours after receipt of the samples at the OADDL. The reference positive and negative controls for the CDV FAT were on a FAT substrate slide (12 well, canine distemper virus, raccoon isolate MA/84, grown in mink lung cells, catalogue number SLD-IFA-CDV; VMRD, Inc., Pullman, WA). The positive reference standard for the CDV FAT was positive in no more than 30% of the cells in the wells. The level of CDV positivity in the samples was calculated as the number of CDV antigen-positive cells divided by the total number of cells in the fields of observation. The whole slide with 8 wells spotted with sample was evaluated in a zigzag manner. All of the slides were read by a board-certified virologist with >30 years of FAT expertise (from June 1982 to the present) in reading FAT slides of cells and tissues. CDV antigen-positive cells were characterized by apple green fluorescence of inclusion bodies of different sizes in the cytoplasm of the infected cells. Variable-size inclusion bodies are a unique characteristic of CDV positivity. The individual reading the CDV FAT slides was blinded with respect to the clinical outcomes of the cases. Positive and negative controls wells were tested simultaneously with each batch of unknown epithelial samples. The batch of positive and negative controls and the anti-CDV-FITC conjugate vial lots were recorded on a worksheet for each run. Observations were made by fluorescence microscopy, at low and high power. Autofluorescence (yellow staining) was recorded as nonspecific. The reproducibility of the assay was excellent, and 4 to 8 wells with the samples were examined to arrive at a final CDV FAT result.

To evaluate the comparative diagnostic efficacy of the direct CDV FAT on the external epithelial surfaces, we performed the first trial with a shelter in Texas, which routinely submits samples

to a commercial diagnostic laboratory for quantitative CDV RT-PCR testing but euthanizes dogs immediately after the initial suspicion of CDV is made for a dog on the basis of clinical signs. Samples were received on frozen ice packs, by overnight delivery. A total of 13 dogs were used for the first trial. Five dogs had results below the cutoff value for the CDV RT-PCR assay and also had negative CDV FAT results; three had results slightly above the cutoff value (about 1 times above the cutoff value by quantitative CDV PCR testing) and were also negative by the CDV FAT. The dogs that had results much higher than the cutoff value (200 to 9,000 times above the cutoff value) were positive by the quantitative CDV FAT. After the initial comparative evaluation of the CDV FAT, a second study was undertaken with an Oklahoma City small-animal clinic, to evaluate the prognostic value of the CDV FAT on external epithelial surfaces for suspected CDV cases.

Based on an Universal Veterinary Information System (UVIS) laboratory information database search, a total of 34 CDV FAT-positive samples (from 2007 to the present) were also examined by virus isolation. About 77% of the CDV FAT-positive samples were confirmed positive by virus isolation on the Vero canine SLAM cell line (26 positive by virus isolation of 34 CDV FAT-positive samples). CDV-negative samples were negative on the Vero canine SLAM cell line. We observed 100% specificity and at least 77% sensitivity, compared to virus isolation, the standard test for CDV detection. The shipment and storage conditions can adversely affect the recovery of live CDV from clinical samples.

To evaluate the CDV FAT for prognostic value and prediction of the final clinical outcomes, we used suspected clinical cases of CDV ($n = 40$ cases with 54 samples) from a large well-managed clinic in Oklahoma City (from January 2013 to September 2014; second trial). All of the dogs were from Oklahoma City, Oklahoma. After adoption from local shelters to households in the Oklahoma City area, these dogs became sick with respiratory disease and presented to the Neel Veterinary Clinic for treatment. At the shelters, the dogs had received modified live CDV vaccines.

All of these suspected CDV cases were independently evaluated for clinical signs by veterinarians and staff members of the Neel Veterinary Clinic (Oklahoma City, OK) before diagnostic testing using the CDV FAT was performed at the OADDL (Stillwater, OK). These samples were sent to the OADDL for CDV FAT diagnosis on ice packs, using a courier service, to preserve the quality of the samples. At the time of receipt, the condition of the specimens was recorded, as shipment temperature affects the quality of the CDV FAT results. Only samples that were received in satisfactory shipment condition were examined at the OADDL by CDV FAT. The external epithelial surfaces evaluated were conjunctiva, nasal cavity, and genital cavity (prepuce or vagina), using cotton swabs, individually or pooled from the same animal. Some samples were submitted as epithelial smears on slides that had been air dried at room temperature for 15 min and were shipped in plastic slide mailers on frozen ice packs. The swab samples were submitted in red-top tubes in normal saline (0.9% sodium chloride), chilled on ice packs. For processing of the samples, the epithelial cells were washed once with phosphate-buffered saline (pH 7.2), and the cells were centrifuged at 3,000 rpm for 10 min. Washed pelleted cells were spotted on charged slides (HTC Super-cured slides; Cel-Line/Erie Scientific). After settling of the epithelial and other mucosal cells on etched circles, the slides were air dried for about 15 min to attach the cells. After 15 min of fixation in methanol-acetone, the cells were stained for 30 min with polyclonal

TABLE 1 Detection of canine distemper virus antigen in cases of respiratory disease at the Neel Veterinary Clinic (Oklahoma City, OK)^a

Serial no.	Accession no.	Specimen type ^b	Result	Clinical outcome ^c
1	13090015	SLI	Inconclusive	U
2	13040465	SLI	Negative	E
3	13040606	Brain	Negative	E
4	13042005	SLI	Negative	W
5	13050379	SLI	Negative	W
6	13051661	CONJ	Negative	W
		NASSMR	Negative	
		PRE	Negative	
7	13060845	CONJ	Negative	W
		NASSMR	Negative	
		PRE	Negative	
8	13060948	SLI	Negative	W
9	13071073	SLI	Negative	W
10	13071202	SLI	Negative	W
11	13080176	SLI	Negative	W
12	13080712	SLI	Negative	D
13	13101438	SLI	Negative	W
14	13120506	SLI	Negative	W
15	13121045	SLI	Negative	W
16	14010078	SLI	Negative	W
17	14011493	SLI	Negative	W
18	14020338	NASSMR	Negative	W
		PRE	Negative	
19	14021350	CONJ	Negative	W
		NASSMR	Negative	
		PRE	Negative	
20	14041672	SLI	Negative	D
21	14050849	SLI	Negative	W
22	14051071	Nasal	Negative	W
23	14051441	CONJ, nasal, genital	Negative	W
24	14051445	CONJ, nasal, genital	Negative	W
25	14060025	Nasal	Negative	E
26	14070251	NASSMR	Negative	W
27	14070315	Nasal, genital	Negative	W
28	14080236	Nasal	Negative	W
29	14080775	SLI	Negative	W
30	13060406	Lung	Negative	D
31	14020832	CONJ	Negative	D
		NASSMR	Negative	
		VAG	Negative	
32	13041370	PRESWAB	Strong positive	E
33	14040424	CONJ	Strong positive	W
		NASSMR	Strong positive	
		PRE	Strong positive	
34	13010489	Tongue	Positive	E
35	13041370	NASSMR	Positive	E
		SBC	Positive	
36	13050285	CONJ	Positive	W
		NASSMR	Positive	
		VAG	Positive	
37	14020338	SBC	Weak positive	W
38	14051294	Nasal, CONJ	Weak positive	E
39	14080099	NASSMR	Weak positive	W
40	14051071	CONJ	Suspect	W

^a A total of 40 cases and 54 samples were examined at the OADDL. Negative indicates that no CDV antigen was detected by the CDV FAT. Positive results and the degree of positivity with the CDV FAT were recorded for each case. Specimens were submitted as slides or swabs over frozen ice packs.

^b SLI, slides with smears from swabs of conjunctival, nasal, and genital epithelia; CONJ, conjunctiva; NASSMR, nasal smear; PRE, prepuce; VAG, vagina; PRESWAB, prepuce swab; SBC, conjunctiva swab.

^c Clinical outcomes were coded by the Neel Veterinary Clinic as follows: E, euthanasia; W, well and clinically normal after symptomatic therapy for respiratory disease at the clinic; D, died; U, unknown (client could not be reached after repeated attempts; serial number 1).

anti-CDV-FITC conjugate (VMRD, Inc., Pullman, WA). After washing of the unbound FITC conjugate, the slides were counterstained with Evans blue for 15 min. The CDV antigen-positive cells exhibited apple green fluorescence, and CDV antigen-negative cells were brick red due to counterstaining with Evans blue. CDV FAT controls were stained simultaneously with each test run, under similar conditions using the same vial of the polyclonal anti-CDV-FITC conjugate (VMRD, Inc., Pullman, WA).

The feedback received from the clients and owners of the dogs about the clinical outcomes of the cases tested by direct CDV FAT after supportive treatment for respiratory disease at the Neel Veterinary Clinic (Oklahoma City, OK) is shown in Table 1. One sample was inconclusive due to insufficient cells due to improper sample collection ($n = 1$, serial number 1). This sample (serial number 1) was excluded from prognosis calculations.

There were 30 dogs for which the CDV FAT results were negative (Table 1). Of the 30 dogs with negative CDV FAT results, 24 dogs recovered, three were euthanized as requested by the clients, and three died. Thus, the response to symptomatic therapy was very good, dogs with negative CDV FAT results had good recoveries, and test results had good prognostic value (24 dogs recovered of 30 that tested CDV FAT negative, i.e., FAT-negative group survival prognosis of 80%).

Nine dogs tested positive or suspect with the CDV FAT in the study. Of the 9 CDV FAT-positive dogs, 4 were euthanized at the request of the clients as their clinical conditions worsened, and 5 dogs recovered clinically. The prognostic index for the CDV FAT-positive group of vaccinated dogs was much lower (FAT-positive group survival prognosis of 55%). CDV disease, if it progresses beyond the initial respiratory disease, is fatal in unvaccinated dogs, based on OADDL cases. Dogs that do not respond early to therapy progress to neurological disease and are euthanized. Current commercial CDV vaccines are effective and provide rapid onset of protective immunity (6). CDV FAT-positive dogs needed to be housed in isolation at the clinic to prevent transmission of the CDV to other dogs. Sick dogs were sent home after symptomatic treatment, to recover from respiratory disease. The CDV FAT was found to be a useful supportive tool for veterinarians in decision-making regarding recommending treatment for suspected CDV cases. The final outcomes of therapy for CDV-vaccinated, sick dogs were better for CDV FAT-negative cases (about 80% surviving) than for CDV FAT-positive cases (55% surviving); however, the differences in prognostic outcomes for the group that tested negative by CDV FAT versus the group that tested positive by CDV FAT were found to be not statistically significant ($P > 0.05$). However, use of the CDV FAT on external epithelia helps avoid unnecessary euthanasia of dogs. Moreover, veterinarians can more correctly inform clients about the potential prognosis for suspected CDV cases based on this study.

Based on published literature findings, the CDV FAT has applications in the detection of CDV antigen up to 30 days after experimental (7) and natural (8) infections. However, the effects of recent CDV vaccination and the emergence of genetic variants of CDV (1) on detection by direct fluorescent antibody testing have not been studied. We found that the CDV FAT, unlike detection by RT-PCR testing, does not interfere with recent vaccination up to 4 days after vaccination, based on the histories provided by the clients of the Neel Veterinary Clinic (Oklahoma City, OK). There are significant differences between dogs with modified live attenuated CDV vaccination and natural exposure to wild-type

CDV. The routes of exposure are aerosols to external epithelia in natural CDV infections and parenteral administration for commercial CDV vaccines. The genotype of the CDV vaccine is America-1 (chicken fibroblast-adapted Onderstepoort strain from the 1960s), which is now extinct in nature, and the current carnivore-source wild-type CDV variants in natural exposure are European wildlife, America-2, and Arctic genetic variants (1). The infectivity, pathogenic potential, and level of shedding of attenuated vaccine virus are very low, compared to wild-type CDV variants. CDV genetic variants differ in their predilections for canine cell types (epithelia and/or lymphocytes). Moreover, the specimen type (urine, whole blood, cerebrospinal fluid, or epithelial mucosa) used in the tests can make a major difference in CDV detection.

The FAT on external epithelial surfaces was found to be a useful prognostic tool for CDV diagnosis and avoidance of unnecessary euthanasia of vaccinated dogs with respiratory disease. CDV is highly lethal in unvaccinated dogs (9). Moreover, conjunctival samples have been reported to be useful for detection of CDV early after infection. Because CDV persists in the conjunctival epithelium longer than in other tissues, the former is a sample of choice for diagnosing CDV (10). The direct fluorescent antibody test on external epithelial surfaces was found to be a rapid (within 2 h after arrival in the laboratory), easy, accurate, and inexpensive alternative to CDV RT-PCR testing for recently vaccinated dogs. CDV FAT results were found to be good prognostic indicators and predictors of clinical responses to symptomatic therapy in CDV-vaccinated dogs sick with respiratory disease.

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